THE EFFECT OF SUBSTITUTION OF CYTOPLASMIC ORGANELLES ON THE RESPONSES OF WHEAT TO FOLIAR PATHOGENS

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MASTER OF SCIENCE

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ABSTRACT

Parastagonospora nodorum and *Pyrenophora tritici-repentis* are foliar pathogens of wheat, capable of causing up to 50% yield losses, and reduced grain quantity and quality, by infecting wheat leaves and kernels. The most economical means of disease control has been through the introduction of nuclear resistance genes. Changes in wheat's nuclear-cytoplasmic (NC) interactions by the introduction of alien cytoplasms, can affect disease resistance. In this study, we investigated 32 alloplasmic lines derived from wheat cultivars Selkirk, Chris, and 56-1 (durum), in response to isolates of *P. nodorum*, *P. tritici-repentis*, and four host-specific toxins produced by them. All alloplasmic lines showed no difference from their parental lines with regards to toxin sensitivity. With spore inoculations, alloplasmic lines, SSM0039, SSM0237, and SSM0241, containing either *Aegilops bicornis* or *Ae. longissima* cytoplasm, exhibited increased disease resistance, compared to their parental lines. Two lines were found to be resistant to multiple isolates or pathogens.

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GENERAL INTRODUCTION

In terms of tons produced, wheat (*Triticum aestivum* L.) is the third most important cereal in the world, after maize and rice (FAOSTAT, 2013). Wheat provides a staple food for 35% of the world's population, and provides the highest number of calories and protein from grains to the human diet (IDRC, 2016), making it one of the most important crops grown in the world.

Wheat production may be affected by biotic and abiotic stresses. Biotic stresses, such as plant pathogens, cause up to $14 billion in losses worldwide (Oerke et al., 1994). Abiotic stresses, such as drought, salinity, and extreme temperatures, may cause yield losses of over 50% on wheat (Bray et al., 2000). Wheat breeders have worked to increase resistance against both biotic and abiotic stresses through the selection of nuclear genes. An untapped possible source of genetic variation in wheat is the cytoplasmic genome, and the subsequent interactions between the nuclear and cytoplasmic genomes. Nuclear-cytoplasmic (NC) interactions are critical to all eukaryotic organisms. In plants, NC interactions influence several processes, including ATP production, gene regulation, and morpho-physiological functions (Tsunewaki, 1980). Substitution of the cytoplasmic organelles that contain a genome, mitochondria and chloroplasts, with those from other closely related grass species, while maintaining the original nucleus, may lead to the gain of favorable traits in wheat, such as disease resistance and increased biomass.

To study NC interactions in common wheat (*T. aestivum*), cytogeneticists and breeders have created numerous alloplasmic wheat lines, in which the original cytoplasmic genome has been substituted with the cytoplasm from a related grass species, such as *Triticum* or *Aegilops* L. (Busch and Maan, 1974). Changes most commonly observed by introducing an alien cytoplasm include delayed heading, changes in the leaf color, decreased vigor, and male sterility (Fukasawa, 1959; Tsunewaki, 1980). In addition, alloplasmic wheat lines can vary in their

disease responses against foliar pathogens, showing either increased or decreased resistance. Washington and Maan (1974) conducted experiments with Chris, Selkirk, and 56-1 durum alloplasmic lines, in which they found that 56-1 alloplasmic lines were more tolerant to leaf rust disease, caused by *Puccinia recondita* Dietel & Holw., and showed less uredial development, when compared to the parental line 56-1.

The purpose of this project was to evaluate the effect of NC interactions on the response of alloplasmic wheat lines to disease and toxins produced by two foliar pathogens, *Parastagonospora nodorum* (Berk.) Quaedvlieg, Verkley & Crous, which causes stagonospora nodorum blotch (SNB), and *Pyrenophora tritici-repentis* (Died.) Drechs., which causes tan spot, in comparison to their parental (euplasmic) lines. Most of the alloplasmic lines used in this study were backcrossed at least 10 times to ensure that the nucleus was similar to the parental nuclear genome (99%). Any resulting difference in the response of the parental and alloplasmic lines will likely be due to the cytoplasmic genome. A unique NC interaction may provide a source of resistance against these pathogens, which would help breeders in developing more resistant wheat cultivars.

CHAPTER 1: LITERATURE REVIEW

Wheat history, production, and issues, globally and locally

Wheat History

Wheat has been the most stable food source on earth. For the last 8000 years, wheat has been the main food crop grown in Europe, West Asia, and North Africa. Wheat, along with rice and maize, is one of the most important cereal crops grown in the world (Curtis et al., 2002). Wheat's origin, as determined by the genetic relationships among all wheat types, was in the southeastern part of modern day Turkey (Dubcovsky and Dvorak, 2007). Wheat was one of the first grains cultivated by farmers, and was selected by them from among wild populations for characteristics such as increased yield and the possession of other favorable traits (Shewry, 2009). Wheat cultivation expanded throughout Anatolia and then into Greece by 8000 BP. It then expanded north into the Balkans and the Danube Valley by 7000 BP. By 5000 BP, it had spread to the United Kingdom and Scandinavia, and then from Iran into central Asia, reaching China by about 3000 BP and into Africa, via Egypt. In 1529, it was carried by the Spaniards to North and South America, Mexico, and then finally to Australia in 1788 (Feldman, 2001). Wheat cultivation now covers more acreage worldwide than any other food crop.

Global Wheat Production

Currently, there are two types of wheat widely produced, based on chromosome number count. Hexaploid wheat (*T. aestivum*), commonly known as bread wheat, and tetraploid wheat (*T. durum* Desf.), also known as durum wheat, which is used to make pasta and other products. Over 700 million tons of wheat are produced each year (FAO, 2015a). About 95% of worldwide wheat production is bread wheat, while durum represents only 5% (Shewry, 2009). In 2015/16, the top three wheat producers in the world were the European Union, with 156.53 million tons,

China, with 126.21 tons, and India, with 95.85 tons (USDA-ERS, 2016a). In that year the United States ranked fourth in worldwide production, with 55.15 million tons valued at over $11 billion (USDA-ERS, 2016a). In terms of total wheat exports, the European Union is the largest exporter with 32 million tons, the United States is second, with 23.5 million tons, followed by Canada exporting 21 million tons (FAO, 2015b). In 2015/16, 54.6 million acres of wheat were planted in the US, with 47.1 million acres harvested. The average yield was 43.6 bushels per acre, producing a total supply of 2,924.1 million bushels (USDA-ERS, 2016b).

Wheat Production in the United States

In the United States, six classes of wheat are grown: Hard Red Winter Wheat (HRWW), Hard Red Spring Wheat (HRSW), Hard White Wheat (HWW), Soft White Wheat (SWW), Soft Red Winter Wheat (SRWW), and Durum. They are grown in different regions of the US, based on the environment, growing season, intended use, and other characteristics (USDA-ERS, 2016c). In the US, HRWW accounts for 40% of the total wheat production, and is mainly used in the production of bread flour. HRSW, which accounts for 20% of wheat production, has the highest protein content, and is also used in bread-making. SRWW accounts for 15-20% of wheat production, contains medium protein content, and is used in making cakes and cookies. SWW accounts for 10-15% of total production, has low protein content, and it's used for making Asian-style noodle products and cereals. HWW is a new class, contains medium to low protein content, and is mainly used in making different types of breads. Lastly, durum wheat is the least produced, accounting for only 3-5% of total wheat production, contains high protein content, and is mainly used for making pasta (USDA-ERS, 2016c).

Wheat Production in North Dakota

In 2015, North Dakota produced 370 million bushels of wheat, making it the largest wheat producing state in the US, followed by Kansas, which produced 321 million bushels (USDA-NASS, 2016). One-fourth of North Dakota's total land area is used to grow wheat, and it produces about one-third of the state's total farm income (NDWC, 2016). Moreover, ND is the leading producer of HRSW and durum wheat in the United States. SWW and HWW are also produced in ND, but in smaller proportions. In 2015, ND's HRSW production was 319 million bushels and the state produced 42 million bushels of durum wheat (USDA-NASS, 2016). Due to the harsh winter weather conditions, ND produces little winter wheat, which accounts for only 3-10% of the state's total production (NDWC, 2016).

Issues in Wheat Production Globally and Locally

Wheat production is affected by numerous biotic and abiotic stresses. Environmental conditions, such as extreme temperatures, drought, soil acidity or salinity, and toxicity from minerals, have the ability to reduce the quantity and quality of wheat (Sutton, 2009). Drought has caused major losses in several US states. For example, in 2006 and 2012, drought in Texas caused more than $4.1 billion in yield losses in crops, and of that amount, $243 million in losses were estimated to be just in wheat production (Dreibus, 2014). In addition, safeguarding crops from damage caused by weeds, animal pests, and pathogens, is a major task necessary to maintain the delivery of a high quantity and quality food. There is a high potential for economically significant losses in wheat production due to fungal and bacterial pathogens, viruses, animal pests, and weeds, which are estimated to cause 16%, 3%, 9%, and 23% in yield losses each year, respectively. Globally, weeds are considered the most significant pests (Oerke and Dehne, 2004).

Competition between wheat and neighboring weeds for nutritional resources and space is affected by the morphological traits of both plants, and includes characteristics such as plant height, tillering capacity, and the time from planting to maturity (Worthington et al., 2015). There are several important weeds commonly found in wheat growing areas, including jointed goatgrass (*Aegilops cylindrica* Host), volunteer rye (*Secale cereale* L.), and downy brome (*Bromus tectorum* L.) (Elliott, 2010). In addition, some weeds may serve as alternative hosts for wheat pathogens, such as *P. nodorum*, which can infect different species of *Elymus* or *Leymus* (wild rye and wheat grasses) (Eyal, 1999a). Many insects have also been reported to cause economic losses in wheat growing areas of different regions. For instance, in the Mediterranean and Western Asia, Hessian fly (*Mayetiola destructor* Say.) affects plants by reducing tillering and kernel growth (Duveiller et al., 2007). Pathogens, such as *P. nodorum,* which causes Stagonospora leaf blotch or SNB, *P. tritici-repentis*, causal agent of tan spot, and rust fungi (*Puccinia* spp.), cause major losses in wheat production worldwide. SNB and tan spot have been reported in most of the wheat growing areas of Australia, North America, South America, South Asia, and North Africa (Crook et al., 2012; Friesen et al., 2005; Murray and Brennan, 2009). Both diseases, under optimal conditions, may cause serious yield losses of up to 50% (King et al., 1983; Rees et al., 1982; Sharp et al., 1976; Wicki et al., 1999).

Losses caused by pathogens are of great economic importance, and establishing resistance against these pathogens is the most effective and environmentally safe means of protecting this resource. The introgression of nuclear resistance genes into wheat from other grass relatives has contributed to reducing these losses. For example, *Aegilops sharonensis* Eig is known for its resistance against multiple pathogens, such as powdery mildew, spot blotch, and

leaf and stem rusts. These resistance genes were introgressed into wheat, in order to increase the resistance gene pool against these pathogens (Olivera and Steffenson, 2009).

Introducing an Alien Cytoplasm

Another possible source of genes to improve wheat are in cytoplasmic organelles, which include mitochondria and chloroplasts. The interactions of organellar genomes with the nuclear genome, called nuclear-cytoplasmic (NC) interactions, are an important element in the speciation and survival of all eukaryotic organisms. Normal cell function requires a well-regulated interplay between the nuclear and cytoplasmic genomes (Leon et al., 1998). Plant cytoplasmic genomes are comprised of 120-140 genes in the mitochondria and 95-100 genes in the chloroplast (Schuster and Brennicke, 1994; Sugiura, 1992). In contrast, animal cytoplasms do not have chloroplasts and the mitochondrial genome consists of only 37 genes (22 mt tRNAs, 2 rRNAs, and 13 for proteins) (Boore, 1999). Over evolutionary time, in both mammals and plants, many genes have been transferred from the cytoplasmic genome to the nucleus. This has resulted in about 90% of the proteins required by cytoplasmic organelles needing to be imported into the organelle (Gillham et al., 1994; Leon et al., 1998). Genetic changes in the nuclear and cytoplasmic genomes may occur by point mutations, intramolecular homologous recombination, and stoichiometric alterations (Soltani et al., 2016). Coevolution has resulted in a compatible interaction between the nuclear and the organellar genomes. Incompatible interactions can occur when the cytoplasm of one species is placed into the nuclear background of another. When this occurs, undesirable traits, such as cytoplasmic male sterility, stunted growth, and morphological changes in flowers, may appear (Levin, 2003). Noyszewski et al. (2014) studied the changes that occur in the mitochondrial genome when it is coupled with a new nucleus, as is the case in alloplasmic wheat. They sequenced the mitochondrial genome of an alloplasmic wheat line, and

that of its cytoplasmic donor, *Aegilops longissima* Schweinf. & Muschl., and the nuclear donor, *Triticum turgidum* L. They observed that the mitochondrial genome of the alloplasmic line had been significantly rearranged compared to the cytoplasmic and nuclear donors, with changes in several of the genes due to the rearrangement and appearance of new open reading frames (ORFs).

In humans, mitochondrial dysfunction is believed to be an important contributing factor in many diseases, including bipolar disorder, dementia, Alzheimer's disease, epilepsy, migraine headaches, and strokes. Most of these diseases share a similar dysfunction mechanism, which is related to the production of reactive oxygen species (ROS) (Pieczenik and Neustadt, 2007). In plants, introducing an alien cytoplasm, i.e. creating an alloplasmic line, can cause changes in quantitative and biological traits. Most wheat alloplasmic lines are created via a series of backcrosses using the female parent as the cytoplasmic donor and the male parent as the nuclear donor (Tsunewaki et al., 1996). Changes in the plant's traits under alloplasmic conditions are caused by either the disruption of mitochondrial and nuclear interactions, or of the chloroplast and nuclear interactions, or both. Cytoplasmic male sterility (CMS) is one of the most common results of the alloplasmic state, and it is often the result of the proteins encoded by the mitochondrial genome improperly interacting with the proteins encoded by the nucleus. This mitochondrial dysfunction results in a lack of energy during pollen formation, resulting in CMS (Hanson, 1991; Levings and Vasil, 1995). This has also been demonstrated in alloplasmic CMS tobacco plants, *Nicotiana tabacum* L., where the tobacco nucleus was combined with the cytoplasm from other *Nicotiana* species (Bonnett et al., 1991). In many CMS lines the chloroplast genomes of both parents are similar (Hanson, 1991; Saumitou-Laprade et al., 1994). However, in other alloplasmic organisms, the chloroplast-nuclear interactions are disrupted,

resulting in the lack of proper photosynthesis, which most often results in chlorosis of the plant (Bannerot et al., 1977).

Wheat has the largest collection of alloplasmic lines *in planta*, which makes it the best model to investigate the mechanisms behind NC-interactions. Large collections of alloplasmic wheat lines have been created, containing cytoplasmic materials from other species in genera such as *Triticum, Aegilops, Agropyron,* and *Secale*. For example, the nuclear genome from *T. durum,* durum wheat, was introduced into the cytoplasm of six *Triticum* spp., 14 *Aegilops* spp., one *Haynaldia* sp., and one rye, *Secale cereale* L., via backcrossing (Maan, 1975; Panayotov, 1983; Suemoto, 1983; Tsunewaki et al., 1996).

Alloplasmic wheat lines express different traits than their parental, or euplasmic, line. Euplasmic lines contain the cytoplasm that has coevolved with the nuclear genome (Busch and Maan, 1974). Wheat alloplasmic lines with *Triticum* or *Aegilops* cytoplasms often exhibit changes in vigor, biomass, and yield, especially when there are large genetic differences between the genomes of the cytoplasmic and the nuclear donors (Wilson and Driscoll, 1983). For example, bread wheat with cytoplasm from *Ae. ovata* Roth, *Ae. umbellulata* Zhuk., *Ae. mutica* Boiss., or *Ae. comosa* Sm., show a reduction in vigor. Also, the respiratory activity in bread wheat was reduced when its cytoplasm was replaced by one from *Ae. umbellulata* (Iwanaga et al., 1978).

Changes in resistance and susceptibility to different pathogens have also been observed. Alloplasmic lines of *Triticum aestivum,* Chris and Selkirk, and *T. durum* line 56-1 were evaluated for their reaction to three different races of the wheat leaf rust *Puccinia recondita* (Washington and Maan, 1974). The study was conducted at the seedling stage, and they found that Chris alloplasmic lines were susceptible, or moderately susceptible, to one or more races,

unlike the parental line, which was susceptible only at the adult stage. These lines contained cytoplasmic material from *T. araraticum* Jakubz., *T. timopheevii* (Zhuk.) Zhuk., *T. macha* Dekap. & Menab., *T. boeoticum* Boiss., *Ae. ovata* L., or *Ae. speltoides* Tausch. In contrast, durum alloplasmic lines derived from 56-1, with the same cytoplasmic materials or others, showed more tolerance to the disease than the parental line, and exhibited slower uredial development (Washington and Maan, 1974). In addition, the disease reaction of alloplasmic wheat lines derived from Selkirk, Chris, or Chinese Spring to loose smut, *Ustilago tritici* (Pers.) Rostr., was evaluated. Some lines from Chris and Selkirk showed race-specific susceptibility to the pathogen, unlike the parental lines, while Chinese Spring alloplasmic lines showed reduced resistance compared to the parental line (Dhitaphichit et al., 1989). In another study, an alloplasmic line derived from the cultivar (cv.) Roazon, with the cytoplasm from *Ae. ventricosa*, was evaluated for its disease resistance/susceptibly against eyespot disease, caused by the fungal pathogen *Pseudocercosporella herpotrichoides* (Fron.) Deighton. Partial resistance was seen in the alloplasmic line when compared to the parental line (Dosba and Doussinault, 1978). Keane and Jones, in 1990, studied the resistance/susceptibility of Chris and Selkirk alloplasmic lines, constructed with the cytoplasm from different species of *Aegilops*, to *P. nodorum*. In this study, observations were made of the disease level on the flag leaf and of head infections. They found an increase in susceptibility in all of the alloplasmic lines, compared to their parental line, at the flag leaf stage, except the line with *Ae. variabilis* Eig cytoplasm, which showed resistance, similar to its parental line. At head infection, all Selkirk alloplasmic lines exhibited a reduction in yield, as did its parental line. Interestingly, the lines with *Ae. cylindrica* Host, *Ae. squarrosa* L., and *Ae. ventricosa* Tausch cytoplasms yielded better than the parent Selkirk when it was infected. In this project, our goal is to characterize the effect of NC-interactions on responses of

alloplasmic wheat to diseases and toxins using several isolates and/or races the pathogens which cause SNB and tan spot.

Tan spot disease

History

Throughout the world, tan spot, caused by *P. tritici-repentis*, is an economically significant fungal disease of wheat (Lamari and Bernier, 1989a). The fungus was first described in Germany in 1823, and was again identified in the early 1900s in Europe, Japan and the United States (Diedicke, 1902; Dreschsler, 1923; Nisikado, 1928). However, tan spot outbreaks were not common until after the 1940's. In the 1970s, epidemics were reported in the US, Canada, Australia, and southern Africa (Hosford, 1971; Rees and Platz, 1983; Tekauz, 1976). At that time, it was believed that these epidemics were the result of no-tillage cultural practices, which were used in an effort to decrease soil erosion. Currently, evidence supports the conclusion that the necrosis toxin gene, *Sn/Ptr ToxA* (*P. nodorum/P. tritici-repentis* ToxA), was transferred from *P. nodorum* to *P. tritici-repentis* at that time, making this organism a much more aggressive pathogen (Friesen et al., 2006). The pathogen can cause yield losses by affecting kernel weight, total number of grains per head, leaf area, tiller number, dry matter accumulation, and grain size. Researchers observed the highest yield losses were caused by the pathogen when infection occurred during later growth stages, such as flowering and boot stages (De Wolf et al., 1998; Rees and Platz, 1983; Shabeer and Bockus, 1988). Also, seed quality may be reduced by the appearance of red and dark smudge symptoms, which are also caused by the infection. The reddish discoloration is most obvious in durum wheat seeds, rather than hard red spring wheat, due to the lack of red coloration of the seeds (Fernandez et al., 1994; Valder, 1954). Yield losses

caused by the fungus can reach up to 50% when both favorable environmental conditions occur and susceptible genotypes are used (Shabeer and Bockus, 1988).

Cultural practices, such as zero-tillage and no crop rotation, could increase the disease intensity in wheat production areas. The pathogen can overwinter on wheat residues, suggesting that zero-tillage practices would increase the inoculum quantities, contributing to disease outbreak. Fungicide treatments can decrease the disease pressure, leading to less significant losses. However, the use of such chemicals may harm the environment and increase the cost of production (Bockus and Claassen, 1992; Sutton and Vyn, 1990). Therefore, providing wheat lines with resistance to tan spot is the best way to control the disease in an environmentally friendly and less costly way, and alloplasmic wheat lines may aid this process.

The most recent North Dakota wheat disease survey, conducted in spring 2016, showed tan spot is one of the most common wheat diseases in the state. Disease severity reached 50% in both the southwestern and northwestern production areas of the state (NDSU Extension Service, 2016a). Since the pathogen has the ability to overwinter on wheat residues, controlling the disease at low levels is especially difficult when optimum conditions are available, such as the presence of a previously infected crop, a susceptible variety of wheat is planted, temperatures above 10°C, and wet conditions (NDSU Extension Service, 2016b).

Taxonomy and Morphological Characteristics of *P. tritici-repentis*

Pyrenophora tritici-repentis (Died.) Drechs. was initially isolated in Germany from the grass species *Agropyron repens* L. and classified as *Pleospora trichostoma* by Diedicke in 1902. Later he renamed it *Pleospora tritici-repentis*. In 1923, Drechsler also identified the fungus and named it *Pyrenophora tritici-repentis*. Nisikado, in 1928, isolated the same fungus from wheat and named it *Helminthosporium tritici-vulgaris*. Later, Nisikado placed *H. tritici-vulgaris* into a

group with five different species of *Helminthosporium* under the genus *Drechslera*. In 1962, Shoemaker believed that *Drechslera tritici-vulgaris* (Y. Nisik.) S. Ito was synonymous with *Drechslera tritici-repentis*. Together Shoemaker and Wehmeyer argued whether the name should be changed from *Pleospora* to *Pyrenophora*. Since the sexual form had not been found yet, Wehmeyer suggested keeping it under the genus *Pleospora*. Later, after Shoemaker recovered both sexual and asexual forms of the fungus, it was agreed to place the teleomorphic form under the name *Pyrenophora tritici-repentis. P. tritici-repentis* belongs to Phylum Ascomycota, Class Dothidiomycetes, Order Pleosporales, Family Pleosporaceae, and its anamorphic form is *Drechslera tritici-repentis* (Died.) Shoem., which belongs to the Class Hyphomycetes (De Wolf et al., 1998).

P. tritici-repentis' ascospores (the sexual form) are biseriate at the base, and uniseriate toward the tip of the asci. Ascospores are 47-65 μm long by 20-26 μm wide. The spores are brownish in color and form inside asci. The asci are 170 to 215 μm long by 43 to 50 μm wide. These asci are formed in ascocarps, which are the fruiting bodies (protopseudothecia-type). Ascocarps are dark brown in color, spherical to subspherical, smooth, with diameters between 400 and 500 μm. The beak of the ascocarp has a number of dark-brown hairlike structures, which measure 80 to 120 μm long by 9 μm wide (Ellis and Waller, 1976).Conidia, the asexual form, arise from conidiophores, are cylindrical, slightly curved or straight, and are rounded at the tip. The base segment has a unique snake head or conical shape, and are thin-walled with 1 to 9 pseudosepta. In older conidia the spore is constricted at the pseudosepta, and are 80 to 250 μm long and 14 to 20 μm wide. The conidiophores are in groups of 2 to 3, forming throughout the stomata or the epidermal cells, are either straight or bending, and smooth. They are mid-gray to brown in color, and up to 250 μm long (Ellis and Waller, 1976).

Life Cycle

The infection cycle of *P. tritici-repentis* begins with the primary inoculum, which may be derived from infected seeds, secondary hosts, and/or host residues. The primary inoculum can be in the form of either ascospores or conidia. Infected seeds can transport the pathogen in the mycelial form. Secondary, or alternative, hosts can be a source of inoculum during or after the growing season (De Wolf et al., 1998). There are many other closely related grasses that can also be infected by *P. tritici-repentis*, such as *Agropyron* spp., *Alopecurus arundinaceus* Poir., *Andropogon gerardii* Vitman, *Avena fatua* L., *A. sativa* L., *Bromus inermis* Leyss., *Dactylis glomerata* L., *Echinochloa* spp., *Leymus innovatus* (Beal) Pilg., *Lolium perenne* L., *Phalaris arundinacea* (Parnell) Druce, *Poa* spp., and *Secale cereale* (Ali and Francl, 2001; Andersen, 1955; Conners, 1939; Dennis and Wakefield, 1946; Dickson, 1956; Diedicke, 1902; Drechsler, 1923; Farr et al., 1989; Hosford, 1971; Krupinsky, 1992; Morrall and Howard, 1975; Shoemaker, 1962; Sprague, 1950). After the primary infection takes place, conidia will form after 12 to 15 days, when the conditions include low temperatures ranging from 10° to 16°C, high relative humidity, and extended leaf wetness, and cause secondary infections throughout the growing season (De Wolf et al., 1998). Symptoms on susceptible wheat leaves are oval or diamond-shaped, are tan in color, and are surrounded by yellow borders with dark brown spots in the center. As the disease progresses, these lesions enlarge and can coalesce. On kernels, symptoms appear as reddish discolorations, known as red smudge (De Wolf et al., 1998).

Factors that can affect the initiation of the disease cycle and the disease severity include temperature, light, moisture, plant growth stage, plant genotype, and the race of the fungal isolate (Ciuffetti and Tuori, 1999). When the temperatures reach 20°C, combined with 3 hours of leaf wetness, 65% to 78% of conidia will germinate on both susceptible and resistant cultivars (Larez

et al., 1986). However, when the wetness time is increased to 6 hours, 95% of conidial spores germinate (Hosford et al., 1987). In 1986, Luz and Bergstrom inoculated spring wheat cultivars with different isolates of *P. tritici-repentis* and incubated the plants at different temperatures, with 30 hours of wetness. They found an increase in disease severity and the number of lesions when the temperature ranged from 20°C to 28°C. Leaf age also influences disease severity. In a study of 14 winter wheat genotypes inoculated with *P. tritici-repentis,* the highest disease severity occurred on the oldest leaf as compared to the youngest ones (Cox and Hosford, 1987). Conversely, on resistant wheat cultivars, the disease severity remained at the same level at any leaf age (Lamari and Bernier, 1989a, b).

A diurnal cycle affects the formation of conidiophores and conidia. Cultures of *P. tritici-repentis* kept continuously in the light only form conidiophores, but no conidia. When the cultures were kept continuously in the dark, no conidiophores or conidia were formed. If cultures of *P. tritici-repentis* are treated with a light and dark cycle, conidiophores and conidia formed. This showed that *P. tritici-repentis* sporulation is diurnal, with the conidiophores forming in the light and conidia in the dark (Khan, 1971).

Classification of *P. tritici-repentis* Based on Host-Selective Toxins (HSTs)

Host-selective toxins, or effectors, are important factors in the pathogenicity of many pathogens. Of plant pathogens, these toxins are only known to be produced by fungal pathogens, unlike non-host selective toxins, which may also be produced by bacteria. Another difference between HSTs and non-host specific toxins is that HSTs only appear to be produced by pathogens with a small host range (Walton, 1996). HSTs are considered to have relatively low molecular weights (secondary metabolites) and differ in their chemistry and biological effects. HSTs may be peptides, terpenoids, oligosaccharides, or polyketides, and range from 7 to 30 kDa

(Friesen et al., 2008a). There are several pathogens known to produce such effectors, including *Alternaria* spp., *Cochliobolus* spp., *P. tritici-repentis*, *P. nodorum*, and others. Some isolates of a pathogen may not be able to produce these effectors, which results in their being either avirulent or causing less disease on their hosts. Isolates producing these effectors are capable of causing greater levels of disease, which is why these effectors are considered to be virulence factors (Walton, 1996).

Lamari and Bernier (1989b) first classified tan spot isolates into different pathotypes, based on the symptoms observed on a set of differential wheat lines: Glenlea, 6B365, and 6B662. Symptoms of necrosis or chlorosis observed on the differential lines determined the pathotype of the isolate. Four pathotypes were identified based on the response of the differential lines to the infection. Pathotype 1 isolates produced necrosis on Glenlea, chlorosis on 6B365, and no symptoms on the other differentials. Pathotype 2 isolates only produced necrosis on Glenlea and no symptoms on any of the other differentials, while pathotype 3 isolates produced chlorosis on 6B365 and no symptoms on the other differentials. Pathotype 4 isolates were avirulent and did not produce any symptoms on any of the differentials. This study indicated that both the pathogen and the host contained genes that control the expression of these symptoms. This method of classification was effective until the discovery of several isolates from Algeria that developed chlorosis on wheat genotypes such as Katepwa, on which pathotype 3 isolates did not produce chlorosis. Furthermore, several Algerian isolates were avirulent on 6B365, whereas pathotype 3 isolates were virulent (Lamari et al., 1995).

As a result of identifying these additional isolates, the pathotype classification system, based on symptoms produced on the differentials, was not sufficient to identify all of the virulence patterns. Also, by this time three different toxins had been identified from isolates of *P.*

tritici-repentis. This led to the proposal of a second classification system, using the term race, where the presence of the different host-selective toxins or effectors was used to classify the isolates into races (Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). Several differential lines have been identified to determine the races. The following four are one example: Glenlea, ToxA sensitive, Tox B and C resistant; 6B662, ToxB sensitive, Tox A and C resistant; 6B365 ToxC sensitive, Tox A and B resistant; Salamouni, Tox A, B, and C resistant. The classification of the races, and the effectors expressed by each race, is indicated in the table below along with the observed responses of the differentials (Table 1.1).

Table 1.1. Designation of *P. tritici-repentis* races and the response of the differential lines.

Toxin[a]				**Differential[b]**				
Race	ToxA	ToxB	ToxC	Race	Glenlea	6B365	6B662	Salamouni
1	+	-	+	1	nec	chl	-	-
2	+	-	-	2	nec	-	-	-
3	-	-	+	3	-	chl	-	-
4	-	-	-	4	-	-	-	-
5	-	+	-	5	-	-	chl	-
6	-	+	+	6	-	chl	chl	-
7	+	-	+	7	nec	-	nec	-
8	+	+	+	8	nec	chl	chl	-

[a] + = toxin produced, - = no toxin produced.
[b] nec = necrosis, chl = chlorosis.

This race classification, based on the presence or absence of known host-selective toxins (HSTs), was more accurate than the previous system and allowed for the addition of races as new toxins were identified. However, in 2010, the identification of *P. tritici-repentis* isolates from Arkansas, that produced necrosis on the race two differential Glenlea but did not possess the gene for Ptr ToxA, indicated the presence of an additional toxin and races, and the need to identify new differential lines (Ali et al., 2010).

Nomenclature of *P. tritici-repentis*' HSTs

In 1998, the tan spot community met and standardized the nomenclature for the toxins found in *P. tritici-repentis*. It was decided that the toxins would be named in order from A to Z. The author would have to demonstrate that a new toxin was not previously described, demonstrate specificity, and determine the association of the toxin with disease development (Ciuffetti et al., 1998).

Currently, three host specific toxins have been identified in *P. tritici-repentis*: Ptr ToxA, Ptr ToxB, and Ptr ToxC, although several additional toxins have been alluded to. Two of the toxins, Ptr ToxA and Ptr ToxB, are proteins which have been purified and cloned. Ptr ToxC is not a protein and has only been partially purified (Effertz et al., 2002). As stated above, isolates are classified into different races based on their production of these toxins, which are determined by the type of symptoms given on a set of differential lines. To date, there are eight different races of *Ptr* (Table 1.1).

Resistance Mechanisms of Necrotrophic Pathogens

The interaction between a pathogen and a host results in either resistance or susceptibility. These outcomes are controlled by specific genes that are found in both the pathogen and the host. In Flor's gene-for-gene hypothesis, it is stated that the interaction between a biotrophic fungal pathogen, such as a wheat leaf rust, and its host is controlled by pathogen effectors, produced by avirulence genes, and the proteins produced by the dominant host gene, which are known as *R* genes (resistance genes). Recognition between the compounds/proteins encoded by these genes results in a resistant reaction only when both genes are dominant (an incompatible interaction). However, when any one of the genes is recessive, the outcome is a susceptible reaction (compatible interaction) (Flor, 1956). In contrast, in the interactions between

necrotrophic pathogens and their hosts, the interaction of a molecule produced by the pathogen with the host protein results in a compatible interaction and disease. This is known as the inverse gene-for-gene model (Friesen et al., 2008a; Lamari and Strelkov, 2010; Strelkov and Lamari, 2003; Wolpert et al., 2002). Pathogens, such as *P. tritici-repentis* and *P. nodorum,* which produce HSTs, such as Ptr/SnToxA, Ptr ToxB, Ptr ToxC, and SnTox1-7, induce disease when their toxins interact with their respective host gene products. These gene loci also are known as susceptibility loci when they condition for sensitivity. To date, there have been three host susceptibility genes identified in the *P. tritici-repentis*/wheat pathosystem, with some of these genes having been cloned and characterized. In the *P. nodorum/*wheat pathosystem, there have been eight host susceptibility genes identified whose proteins interact with the HSTs produced by the pathogen.

Identification and Characterization of *P. tritici-repentis'* Toxins

Ptr ToxA- *Tsn1* interaction

The presence of secreted toxins that produce the observed symptoms similar to the disease was demonstrated by Tomás and Bockus (1987). They showed that culture filtrates from different isolates could produce either necrosis or chlorosis, or both, depending on the wheat cultivar and isolate used in the assay. In 1989, Ballance et al. published the purification of a proteinaceous necrosis inducing toxin. SDS polyacrylamide gel electrophoresis indicated a molecular mass of 13.9 kDa. They demonstrated that the toxin was heat stable, inactivated by reduction with dithiothreitol, indicating a disulfide bond is necessary for function, and that the toxin was relatively protease resistant. Subsequently, Tomás et al. (1990) published a similar purification process for a proteinaceous necrosis toxin of similar size, 14.7 kDa, which was also sensitive to reducing compounds. Unfortunately, the isolation procedures described in these

papers were difficult to reproduce, and only a limited amount of toxin could be isolated. In 1995, Tuori et al. published an alternate purification procedure that was capable of producing larger quantities of the protein for their research. In their study they established that the toxin had a lower molecular mass than previously reported, 13.2 kDa, but they found that antibodies to their toxin also precipitated the toxin purified by Tomás et al. (1990). They also identified additional potential toxins within their culture filtrates. Using the same isolate used by Ballance et al. (1989), Zhang et al. (1997) isolated a necrosis toxin of the same size as reported by Tuori et al. (1995), and sequenced a majority of the protein by Edman sequencing. Based on circular dichroism spectroscopy and the amino acid sequence, they predicted the secondary structure was two anti-parallel beta sheets stacked on each other. Zhang et al. (1997) also analyzed the sequence for possible phosphorylation sites and suggested that the tripeptide arginyl-glycyl-aspartate (RGD) may be of importance in its function. It had previously been shown that the RGD sequence was important for proteins interacting with integrins found in the plasma membrane, and that integrins were involved in signaling into the cell. The results from these studies suggested that all four groups had isolated the same protein.

Concurrent with the subsequent purification and characterization of the toxin protein, groups were isolating the gene encoding the toxin. From cDNA, Ballance et al. (1989) cloned the 565 bp *ToxA* gene, with 534 bp encoding a protein of 178 amino acids. They predicted a signal sequence of 16 amino acids, with a pro-sequence from amino acid 16 to 50 or 51. Ciuffetti et al. (1997) cloned the nuclear *ToxA* gene, which was 585 bp, containing one intron in the protein coding region, and showed it was present in a single copy. They predicted the toxin consisted of 178 amino acids, with a signal sequence of 22 amino acids, a 38-39 amino acid pro-sequence, and a 117-118 amino acid mature protein. The pro-sequence was suggested to be necessary for

folding, since removal of it in expression systems resulted in no active toxin, and the toxin could not be refolded after denaturation with a reductant. Both the signal and pro-sequence are removed prior to secretion. Kwon et al. (1996, 1998) demonstrated through inhibitor studies that the toxin's function required transcription, translation, and a proton pumping ATPase. It had been shown previously that ToxA was ineffective at low, 4°C, and high, >30°C, temperatures. Kwon et al. also demonstrated that this was due to changes in the plant rather than the toxin.

The *Ptr ToxA* gene has been detected in other foliar pathogens. The gene was found in *Pyrenophora teres*, the causal pathogen of spot form net blotch on barley (Leisova-Svobodova et al., 2010). Sequence variation at the *ToxA* locus was similar between *P. tritici-repentis* and *P. teres*. The *ToxA* gene similarities between both pathogens can be explained by horizontal gene transfer that happened through conidial anastomosis, which occurred in the 1980s (Leisova-Svobodova et al., 2010). Likewise, there has been a horizontal gene transfer of the *ToxA* gene between *P. nodorum* and *P. tritici-repentis* (Friesen et al., 2006). The transfer of this gene is thought to have happened around 1940, when the host was infected with both pathogens, and genetic interchange occurred through conidial anastomosis tubes. This timeline is supported by the fact that prior to this time *P. tritici-repentis* was only reported as a saprophyte, or non-serious pathogen, in grasses or wheat, and the symptoms were seen as chlorosis only with no necrosis (Friesen et al., 2006). Genetic analysis of the gene sequences from a worldwide collection of *P. nodorum* and *P. tritici-repentis* found that all copies of the *Ptr ToxA* gene in *P. tritici-repentis* were identical, but in *P. nodorum* there was significant diversity indicating that it was present in *P. nodorum* for a significantly longer time than in *P. tritici-repentis* (Friesen et al., 2006).

The current model for the mechanism of action of Ptr ToxA begins with the toxin binding to the plasma membrane. It is then taken up into the cell and transferred by an unknown

mechanism to the chloroplast. Once within the chloroplast, the toxin binds to a protein, which results in the inhibition of the electron transport chain, and the generation of reactive oxygen species. The activity of the reactive oxygen species damages the cells membranes and proteins, resulting in necrosis. This process is light dependent and is affected by temperature.

Initial mutational studies (Meinhardt et al., 2002) found that the RGD tripeptide was required for toxin activity. If any of these amino acids were modified, toxin activity was lost. Previously, in studies of other toxins, it had been found that the RGD motif is used to bind to cell membranes through integrins in the plasma membrane. Additional mutational studies indicated that several additional amino acids affected the function of the toxin (Manning et al., 2004, 2007; Sarma et al., 2005; Tai et al., 2007). The idea that a membrane bound receptor was present was also supported by experiments where differing concentrations of the toxin were infiltrated into the leaves (Manning et al., 2007). When dilute solutions of the toxin were used, the necrosis was near the infusion point, but did not cover the whole region infiltrated. As the concentration of the toxin was increased, the area of necrosis increased until it completely covered the infiltration area. This suggested a strong binding of the toxin to a receptor which would concentrate the toxin near the infusion site until the binding sites nearest the infusion point were saturated.

The structure of Ptr ToxA was determined to be similar to fibronectin type II, with two overlapping beta sheets, and the RGD motif was found on the end of a solvent exposed loop that protruded from the protein (Sarma et al., 2005). The amino acids that were shown to affect toxin activity were on or near this loop or caused structural changes.

Kwon et al. (1998) demonstrated the need for protein synthesis using electrolyte leakage experiments. They found that treating the cells with cyclohexamide, a protein synthesis inhibitor, inhibited electrolyte leakage. Treatment of leaves with the toxin, combined with cordycepin and

α-amanitin, which are transcription inhibitors, reduced the degree of electrolyte leakage. Also, treatment with vanadate, an inhibitor of membrane bound proton pumping ATPases, indicated that energy stored as a proton gradient in the plasma membrane was important for the toxin's function.

Uptake of the toxin into the cells was demonstrated by Manning and Ciuffetti in 2005. In their experiments leaves were infiltrated with ToxA, and after incubation for varying lengths of time, the leaves were infiltrated with a solution of proteinase K to digest any toxin remaining outside of the cells (Manning and Ciuffetti, 2005; Manning et al., 2007). They found that if the toxin is present for two hours prior to the proteinase K treatment, the toxin induced necrosis, indicating that the toxin was taken up by two hours after infiltration. Using western blot analysis, they demonstrated that in resistant plants the toxin was digested, even after two hours incubation, and that in sensitive plants the toxin was protected from digestion.

ToxA was found to be localized to the chloroplast in experiments where green fluorescent protein labeled ToxA was infiltrated into the leaves and the cells studied by confocal microscopy (Manning and Ciuffetti, 2005). In this study, they found that ToxA accumulated in the chloroplast. Yeast two hybrid studies found that ToxA bound to a chloroplast matrix protein that was given the name ToxA binding protein (TOXABP1) (Manning et al., 2007). Gene silencing experiments of this protein resulted in reduced ToxA sensitivity (Manning et al., 2010). A subsequent yeast two hybrid study found that ToxA binds to plastocyanin, a component of the electron transport chain (Tai et al., 2007). Recently, Lu et al. (2014) also found that ToxA interacts with a pathogenesis related protein, PR-1-5, and that this protein was differentially expressed in varieties that are sensitive and insensitive.

All attempts to discern the ToxA pathway by mutation of the host indicate a single toxin sensitivity locus, *Tsn1*, which was the same loci that was previously identified by crossing studies, is located on chromosome 5B, and has been cloned and sequenced (Faris et al., 1996). It is a cytoplasmic soluble protein that contains the three motifs which are most often associated with resistance genes, a nucleotide binding site, a leucine rich repeat region (NBS-LRR), and a serine-threonine kinase domain (Eitas and Dangl, 2010; Faris et al., 2010). The transcription of this protein is regulated by sunlight, which explains the necessity of light for toxin activity.

Ptr ToxB- *Tsc2* interaction

Ptr ToxB, a second proteinaceous HST produced by this fungus, induces chlorosis in sensitive wheat lines. ToxB was first identified and characterized from culture filtrates of a race 5 isolate (Orolaza et al., 1995). It was later purified by Strelkov et al. in 1998 and then cloned by Martinez et al. in 2001. Similar to ToxA, ToxB is a small secreted protein, 6.5 kDa, with 4 cysteine residues (Martinez et al., 2001; Strelkov and Lamari, 2003). The ORF of ToxB is 261 bp in length and translates into an 87-amino acid pre-protein with a 23-amino acid signal peptide. Unlike *ToxA*, the *ToxB* gene is present in multiple copies (1-10 estimated) (Lamari et al., 2003; Martinez et al., 2001; Strelkov et al., 2006), and the genes were found on at least two different chromosomes. However, all copies identified in virulent strains were identical. In some non-virulent isolates the nonfunctional *toxb* gene is present but is not expressed at a level that will induce symptoms (Martinez et al., 2004; Strelkov and Lamari, 2003). Although the protein produced by this gene is 86% homologous to the functional *ToxB*, it produces little chlorosis (Kim and Strelkov, 2007).

The *ToxB* homolog found in race 3 isolates, *toxb*, shows differences in the upstream flanking sequence, which alters the putative start codon and increases the size of the putative

secretory signal to 41 amino acids. If the transcription is not affected, and the signal sequence is properly removed, it will result in the production of an identical mature ToxB protein (Strelkov et al., 2006). There are several possible reasons for the lack of ToxB production by race 3 isolates. One is that the changes in the signal peptide are such that it may fail to direct ToxB to the secretory pathway, or that inaccuracy in the secretory pathway may result in alteration of protein folding. Lastly, the lack of multiple copies of *ToxB* may result in too little toxin being produced (Ciuffetti et al., 2010; Strelkov et al., 2006).

It was suggested that the virulence of ToxB producing isolates is related to the copy number of the gene (Amaike et al., 2008; Martinez et al., 2004; Strelkov et al., 2006). To test this theory, race 4 isolates, which are avirulent, were transformed with the *ToxB* gene, which was created using the *ToxA* promoter (Ciuffetti et al., 1997). All transformed isolates were compared to a race 5 isolate for the number of gene copies by Southern blot analysis, and the virulence was determined from inoculations. Race 5 isolate DW7, which maintains multiple copies of *ToxB* and is highly virulent, and race 4 isolate SD20, which only has one copy of *ToxB* and encodes for an inactive toxb protein, were used as controls for this experiment. The transformed isolates of race 4, which with their higher numbers of the *ToxB* gene, showed greater symptoms on the sensitive wheat line Katepwa, when compared to the control isolates. They concluded that virulence of *P. tritici-repentis* isolates with the *ToxB* gene is related to the number of copies of this gene (Ciuffetti et al., 2010).

Several *ToxB* homologs have been found in other genera, including *Cochliobolus*, *Alternaria*, and other species of the genus *Pyrenophora,* such as *Pyrenophora bromi* (Died.) Drechsler, which causes brown leaf spot on smooth bromegrass (Andrie et al., 2008). The homolog *Pb ToxB* gene found in *P. bromi* isolates may be present as a single copy or multiple

copies. Many of these homologs have been cloned from different isolates of *P. bromi*, and show variations in the ORFs within and between isolates. The proteins are expressed at low levels and are 80% similar to *ToxB* and *toxb*. When Pb ToxB was infiltrated into bromegrass leaves, symptoms of chlorosis were not seen. However, when infiltrated into sensitive wheat cultivars chlorosis symptoms developed. This suggests that *P. bromi* may be a potential pathogen in wheat and would help to explain the diversity of this toxin among *Ptr* races and isolates (Ciuffetti et al., 2010).

Although the mode of action of ToxB is not clear, it appears that the toxin has the ability to damage the chloroplasts of sensitive wheat cultivars, similar to ToxA. Current evidence indicates that ToxB is an apoplastic effector (Figueroa et al., 2015). Most apoplastic effectors are small protease resistant molecules with a high quantity of cysteine residues that form disulfide bridges, which help to protect the protein from the high acidity and protease activity in the apoplastic region (Felle, 1998; Hückelhoven, 2007; Joosten and de Wit, 1999; Stergiopoulos and de Wit, 2009; Thomma et al., 2005). Structural analysis using mass spectrometry and nuclear magnetic resonance (NMR) showed that the four cysteine residues located in ToxB create two disulfide bridges, which are essential for ToxB chlorosis activity. Nyarko et al. (2014) proposed that one of the differences between Ptr ToxB and Ptr toxb was the ease of reducing the disulfide bonds. They investigated this using reducing and non-reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE). Numbering from the amino terminus of the mature protein, when the valine 3 residue next to cysteine 2 was replaced with threonine, as is the case in toxb, the disulfide bonds were more susceptible to reduction and there was a reduction in the toxin's activity (Nyarko et al., 2014). Also, a recent study has shown that ToxB is more resistant to proteolysis than ToxA (Figueroa et al., 2015). Apoplastic fluid (AF) extracts from insensitive

and sensitive lines were applied to solutions containing ToxB, toxb, and ToxA as a control. Results from SDS-PAGE and western blot analyses showed both forms of the chlorosis toxin, ToxB and toxb, were resistance to protease activity from AF extracts, unlike ToxA which was not. Localization studies of ToxB were performed using Alexa Fluor 488 labeled ToxB. Using a confocal microscope and fluorescently labeled ToxB, it was shown that ToxB localized to the apoplastic region in both sensitive and insensitive wheat cultivars, but did not appear in the chloroplasts (Figueroa et al., 2015). Activity assays of the fluorescently labeled ToxB showed that it retained its activity. Control experiments using ToxA labeled with the same dye showed its transport to the chloroplast, as seen before. Another difference between ToxB and ToxA is the effect of their concentration on the infiltration zone. For ToxB, the reduction of concentration affects the intensity of the symptoms produced on sensitive leaves, but does not affect the size of the infiltration area, whereas ToxA at lower concentrations does not decrease the intensity of necrotic symptoms, but does affect the size of the necrotic lesion. This may be explained by the presence of high or low compatibility receptors for both toxins. Highly compatible receptors at infiltration points would result in concentrating the effect at one point, with low compatibility receptors resulting in uniform distribution of the toxin (Figueroa et al., 2015).

Aboukhaddour et al. (2012) suggested that ToxB may be involved in the early stages of infection, since silencing ToxB reduced symptoms of chlorosis and the formation of appressoria. The nature and specific features of ToxB, such as existence in multiple copies, and the presence of toxb and other homologs in other genera or species of Ascomycetes, suggest that this toxin has multiple functions, not just creating or promoting chlorosis on sensitive wheat cultivars (Amaike at al., 2008; Ciuffetti et al., 2010).

Although the mode of action of ToxB is not known, the host sensitivity gene for Ptr ToxB, *Tsc2*, is located on the short arm of chromosome 2B (2BS) (Friesen and Faris, 2004). The response to ToxB is light dependent and ToxB infiltration results in degradation of chlorophyll (Strelkov et al., 1998). Pandelova et al. (2012) demonstrated that the presence of ToxB resulted in transcriptional alterations in many genes, including WRKY transcription factors, receptor like kinases (RLKs), pathogenesis related proteins (PRs), phenylpropanoid pathway proteins, and proteins in the jasmonic acid synthesis pathway.

Ptr ToxC- *Tsc1* interaction

Another HST known to be produced by *P. tritici-repentis* is ToxC. This toxin causes chlorosis on sensitive wheat lines, and is produced by race 1 and 3 isolates. In 1995, Lamari and his group found that *Ptr* isolate 78-62 created chlorosis symptoms on wheat cultivar 6B365, which were not induced by ToxB. This showed that *Ptr* isolate 78-62 secreted another toxin that was not yet characterized. Several subsequent reports also identified this uncharacterized toxin (Ciuffetti et al., 1997; Faris et al., 1997; Lamari et al., 1995; Orolaza et al., 1995). The ToxC sensitivity gene, *Tsc1*, was determined by quantitative trait analysis to be on chromosome 1A (1AS) (Faris et al., 1997). Effertz et al. (2002) first characterized ToxC as a low molecular weight, nonionic, polar, non-proteinaceous toxin. As such, its isolation and characterization has been significantly more difficult than the other two toxins, which has resulted in much less known about its mode of action.

Stagonospora nodorum blotch disease

History

Stagonospora nodorum blotch disease, caused by *P. nodorum*, is one of the major foliar diseases affecting wheat production. The disease has been reported in all wheat producing countries worldwide, including the United States, Australia, Argentina, Austria, Canada, China, Denmark, England, France, Germany, Italy, Kenya, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland, Tanzania, and Uruguay (Sprague, 1950). The disease, which affects the leaves, heads, and kernels, can create yield losses up to 50% (Eyal, 1981; King et al., 1983). The symptoms start at the lower leaves and lesions develop to red-brown spots which are lens-shaped and surrounded by yellow borders. Infected heads develop blotch symptoms that range from dark brown to dark purple colored with ash gray parts, and may produce low quality shriveled kernels, which are unsuitable for milling (NDSU Extension Service, 2016). Moderate temperatures, constant rain, the type of cultural practices used, the presence of free inoculum, and the presence of susceptible wheat cultivars are all factors that can lead to disease epidemics (Eyal et al., 1987). This pathogen is now known to produce many toxins, all of which contribute to, and increase, the disease severity (Oliver et al., 2012). Primary inoculum can be found in two different forms, either as ascospores or pycnidiospores. Infected seeds, wheat residues, and alternative hosts all can be sources of the primary inoculum. Seed born inoculum can cause infections at emergence (Solomon et al., 2006). Cultural practices such as low-tillage or zero-tillage increase the chances of early emergence of the disease due to contact between the lower leaves and the infected wheat residues (Eyal, 1999a). Therefore, without a one to two year crop rotation, the disease intensity will continue to increase in the field (Bockus and Shroyer, 1998). *P. nodorum* is widely distributed throughout the US, and has the ability to infect different wheat

classes, including hard red spring wheat and durum wheat. Cultivars vary in their response to the disease. In North Dakota, most of the wheat cultivars are susceptible to the *P. nodorum* isolates present. Ali and Adhikari (2008) studied the variation in aggressiveness of 40 isolates of *P. nodorum*, which were collected from different parts of the state. The isolates were tested on two sensitive lines (ND495 and Alsen) and two resistant lines (Erik and Salamouni). Based on lesion type and necrotic leaf area, they found a high degree of variation in isolate aggressiveness. There was also a strong interaction between the isolate tested and the cultivar, suggesting host specificity, which could be controlled by the effectors produced by the pathogens and their interaction with the proteins produced by the hosts (Ali and Adhikari, 2008).

The main management strategy for controlling this disease is to reduce the inoculum present, by using uninfected seeds, crop rotation, and the use of deep tillage of previous crop residues. In addition, the use of resistant wheat cultivars, along with fungicide applications, minimizes the risk of yield losses in the field (Ali and Adhikari, 2008; Eyal, 1999a; Milus and Chalkley, 1997).

The use of resistant cultivars is the most cost-effective and environmentally safe means to control the disease. The selection of resistance genes in past decades has mainly focused on foliar biotrophic pathogens, such as powdery mildew and rusts, which significantly contributed to yield losses. However, this has led to increased yield losses caused by other pathogens, such as *P. nodorum* (Eyal, 1999b). Resistance in the host against a disease can be either qualitative or quantitative. Highly resistant cultivars, showing no disease symptoms, are usually governed by major genes (qualitative); while in moderately resistant cultivars the resistance is controlled by additive genes. Quantitative resistance, which is governed by several genes (Poland et al., 2009), may be expressed at different stages during disease development, including at infection,

colonization, and/or reproduction. Studies have shown a connection between the cultivar height and its resistance to this pathogen, and that the resistance was due to the pleiotropy of certain genes governing several functions (Eyal et al., 1987). Fried and Meister (1987) showed that leaf resistance to this disease was independent from spike resistance. Seedling inoculations showed different results than field experiments, which was explained by an additive-dominance model.

As stated above, using resistant cultivars is the most effective way to protect wheat from pathogens, however, establishing resistance against SNB is complicated by the nature of the pathogen. Molecular markers have been used to establish genetic linkage maps in wheat populations to help in identifying a number of quantitative trait loci (QTL), their chromosomal positions, and their effects on resistance (Liu et al., 2004a). In one study, several QTLs were shown to be associated with resistance against the pathogen at the leaf and spike infection stages. Since the pathogen also has the ability to secrete virulence effectors, or HSTs, this has aided in our understanding of the host-pathogen interactions, and the genes responsible for susceptibility/resistance. Moreover, additional sources of resistance against the pathogen can be found in other related grasses, and can be used in introgression of resistance genes into wheat cultivars, such as the alloplasmic lines of several wheat cultivars (Washington and Maan, 1974).

Taxonomy and Morphological Characteristics of *P. nodorum*

The asexual form of *P. nodorum* was first described by Berkeley in 1845 as a pathogen that affected glumes and nodes, mostly on wheat, and named *Depazea nodorum*. In 1904, the teleomorphic structures, pseudothecia, were found on *Septoria nodorum* cultures, and then was described by Muller in 1952 as *Leptosphaeria nodorum*. Subsequently, the anamorphic form was moved from the genus *Septoria* to the genus *Stagonospora*, and renamed *Stagonospora nodorum* (Castellani and Germano, 1977). The teleomorphic form was then placed under the genus

Phaeosphaeria, and was renamed *Phaeosphaeria nodorum* (Eriksson, 1967; Hedjaroude, 1968). Most recently, based on phylogenetic analysis, the anamorphic form was renamed *Parastagonospora nodorum* (Quaedvlieg et al., 2013). The same study also confirmed the teleomorphic form of the pathogen as *Phaeosphaeria nodorum*.

P. nodorum belongs to Phylum Ascomycota, Class Dothideomycetes, Order Pleosporales, and Family Phaeosphaeriaceae. It produces asexual spores, pycnidiospores, which are contained inside fruiting bodies called pycnidia. The pycnidia are spherical structures with a diameter between 160 to 210 μm. When first formed, the pycnidia are a light brown color, which later darkens. The pycnidiospores are cylindrically shaped with 0 to 3 septa and their dimensions are 14.2-32 by 1.9-4 μm (Eyal et al., 1987). The spores are surrounded by a mucilaginous medium which inhibits wind dispersal and helps to preserve viability during dry weather conditions. The mucilaginous matrix contains proteins and sugars which will absorb moisture and swell under high humidity. This swelling results in extrusion of the spores through the ostiole of the pycnidium (Fitt et al., 1989). The sexual spores, which are ascospores, are formed inside asci, and these asci are formed inside fruiting bodies called pseudothecia. The pseudothecia range in size from 120 to 200 μm and contain 8 asci that are bitunicate with 4 cells, with the second cell from the tip larger than the others. The ascospores are considerably curved, and their measurements are 23-32 by 4-6 μm with 3 septa (Eyal et al., 1987).

Life Cycle

The pathogen has the ability to overwinter as pycnidiospores inside pycnidia, as ascospores inside pseudothecia, or as mycelia inside infected seeds, which can be found in infected wheat residues. These structures, along with other alternative hosts, are all considered sources of primary inoculum. The ascospores have the ability to travel long distances by wind,

while the pycnidiospores are distributed by rain-splash. Ascospores are unrestricted and free to move at low temperatures, during rainfall, and at high relative humidity (Arseniuk et al., 1998). After the primary infection and symptoms are seen, secondary inoculum is produced by pycnidiospores and released, which causes additional infections. *P. nodorum* can complete a life cycle within 10-14 days, with the asexual spores being released 2 to 4 times per growing season. This polycyclic process can result in considerable disease by the end of the growing season (Bathgate and Loughman, 2001).

There are many factors that affect the life cycle and contribute to disease severity in the field; the presence of favorable environmental conditions, aggressiveness of the isolate, use of susceptible cultivars, and the cultural practices used in the field. The optimal temperature range for fungal growth ranges from 20-24°C, and for disease development is from 20 to 27°C. However, the fungus can grow at temperatures between 4 and 32°C (Wiese, 1987). The conditions required for the highest level of spore germination are temperatures between 15 and 25°C with 6 hours of high relative humidity (Scharen and Krupinsky, 1970). The optimal conditions for infection are temperatures ranging from 15 to 25°C and moisture exposure of 12 to 18 hours. The life cycle of the pathogen can be from 7 to 14 days under optimum environmental conditions (Eyal, 1999a; Wiese, 1987).

Cultural practices are another factor that may affect disease severity. Zero-tillage can result in an increase in early onset of the disease because of the infected residues (Eyal, 1999a). The use of healthy seeds for planting plays an important role in decreasing the disease severity. It has been suggested that one infected seed among 5000 plants is sufficient to create an epidemic (Griffiths and Ao, 1980). The pathogen has a wide range of plants that it can infect. Isolates obtained from 11 different plant species were found to cause disease on six different wheat

species of *Triticum*, and also *Agropyron* spp., *Elymus* spp., *Leymus* spp., and *Hordeum jubatum* L. (Eyal, 1999a). In addition, some isolates of *P. nodorum* appear to be specific for either barley or wheat, and while both are morphologically identical, they are genetically different (Cunfer and Ueng, 1999). These alternative hosts, if they are present in or around the field, may serve as sources of inoculum, which can also lead to epidemic disease levels.

P. nodorum's HSTs

The necrotrophic pathogen *P. nodorum* is now known to produce at least eight HSTs, including: ToxA, Tox1, Tox2, Tox3, Tox4, Tox5, Tox6, and Tox7 (Abeysekara et al., 2009; Friesen et al., 2007, 2008b; Gao et al., 2015; Liu et al., 2004a; Shi et al., 2015). These toxins all contribute to disease development by causing death to infected wheat tissue. Toxins produced by the pathogen determine the host range and affect the level of aggressiveness (Eyal et al., 1987). In one study, 95 HRSW cultivars were tested for susceptibility to the *P. nodorum* isolate Sn4, and 67 were found to be susceptible. This isolate was found in North Dakota and produces ToxA, Tox1, Tox2, and Tox3 (Liu et al., 2015). Like *P. tritici-repentis* toxins, the recognition of the *P. nodorum* toxins by their respective receptors in the hosts, results in host tissue necrosis. For each toxin there is a dominant sensitivity gene product that interacts with the toxin, resulting in cell death. These dominant susceptible genes show an inverse gene-for-gene relationship (Wolpert et al., 2002). Interaction between one of the HSTs produced by *P. nodorum*, and the gene providing sensitivity to it, was first identified in 2004 (Liu et al., 2004a), and helped in creating a model for the identification of other HSTs and their interactions with their respective genes products in wheat.

Nomenclature of *P. nodorum's* HSTs

In *P. nodorum*, any HSTs recognized are named as SnToxN, with N representing the next highest number, which is encoded by the gene *SnToxN*; and the host gene which confers sensitivity to it named as *SnnN*. As mentioned previously, when the interactions between the HSTs and their respective sensitivity gene products occurs in the host, it leads to susceptibility. Therefore, the disease severity may vary on different wheat cultivars, based on presence or absence of multiple known or unknown interactions (Oliver et al., 2012).

Identification and Characterization of *P. nodorum's* Toxins

SnTox1- *Snn1* interaction

In 2004, the proteinaceous necrosis toxin SnTox1 was identified and reported to have a molecular mass ranging from 10 to 30 kDa (Liu et al., 2004a, b). Almost 85% of *P. nodorum* isolates investigated to date produce this toxin (Liu et al., 2012). The mature protein contains 100 amino acids, after cleavage of a signal peptide. It has 16 cysteine residues, which insure the stability and activity of the toxin, and the C-terminus contains chitin-binding domains. When treated with proteinase K, SnTox1 activity was reduced. Also, toxin activity was decreased when it was incubated at 50°C (Liu et al., 2004b). This toxin plays an important role during cell penetration, and ultimately causes cell death. Since it has a chitin-binding domain, it protects the fungus from wheat chitinases (Liu et al., 2012). The interaction of SnTox1 with the protein from the wheat susceptibility gene, *Snn1*, is light dependent. When plants are kept in continuous darkness, no symptoms were apparent after two days. However, when the plants were placed under a light/dark cycle for two days, disease symptoms appeared (Liu et al., 2012). *Snn1* was the first sensitivity gene described, and is located on the short arm of wheat chromosome 1B (1BS). The loci is comprised of two members of the NBS-LRR protein family (Liu et al., 2012).

The SnTox1-*Snn1* interaction starts early in infection, within the first 10 h, and results in the upregulation of the PR1-1 gene, thaumatin-like protein genes, DNA laddering, and other classic hallmarks of apoptosis, which are part of the progression of programmed cell death. It was also noted that ROS increased upon treatment of sensitive leaves with SnTox1 (Liu et al., 2012).

SnTox2- *Snn2* interaction

The effector SnTox2 is a small protein ranging in size from 7 to 10 kDa (Friesen et al., 2007). The development of symptoms with this toxin is also light dependent. The mode of action has not yet been clarified, but since it is also light dependent, it may follow a similar mechanism as SnTox1 for inducing cell death. The effects of SnTox1 and SnTox2 were shown to be additive, therefore, when both are present they will cause more severe disease symptoms on sensitive wheat cultivars. *Snn2*, the host gene that confers sensitivity to SnTox2, is located on chromosome 2D (2DS) (Friesen et al., 2007).

SnTox3- *Snn3* interaction

The toxin SnTox3 has been partially purified and has a molecular mass of 18 kDa. The gene encodes for a 230 amino acid pre-pro-protein, with a 20 amino acid signal sequence, 30 amino acids are a predicted pro-sequence, and the remaining amino acids represent the mature protein (Friesen et al., 2008b; Liu et al., 2009). This effector also has 6 cysteine residues that are thought to be involved in forming disulfide bridges, which are critical to the formation of the functional protein (Liu et al., 2009). This toxin is produced by the pathogen during early growth and induces necrosis on infected leaves. Its production is reduced in preparation for sporulation. Unlike the other toxins, SnTox3 is light independent (Liu et al., 2009). The gene encoding this toxin has been found in approximately 60% of *P. nodorum* isolates tested worldwide, however, the exact gene sequence varies among the isolates (Friesen et al., 2008b; Liu et al., 2009). *Snn3*

confers sensitivity to SnTox3 (Friesen, et al., 2008b), and is located on chromosome 5B (5BS). Another study also found a gene conferring sensitivity to SnTox3, located on chromosome 5D (5DS) (D-genome) in *Aegilops tauschii* (Zhang et al., 2011). The study showed that *Snn3-B1* and *D1* were derived from common ancestors. *Snn3-B1* (B-genome) was derived from *Aegilops speltoides* into hexaploid and tetraploid wheat. Compared to the interaction between SnTox3-*Snn3-B1*, the interaction between SnTox3-*Snn3-D1* is stronger and develops extreme necrosis symptoms (Zhang et al., 2011). Another unique characteristic of the interaction between SnTox3-*Snn3* is that it can be affected by the interaction between SnTox2 and the Snn2 protein. The incompatibility interaction between SnTox2-*Snn2* is epistatic to SnTox3-*Snn3*, which means the disease severity would be less in the presence of *Snn2*. This is unlike the compatibility in the interaction of SnToxA-*Tsn1* and SnTox2-*Snn2*, which are additive and together give rise to significantly more disease than each one does alone (Friesen et al., 2008b).

SnTox4- *Snn4* interaction

A fourth effector, SnTox4, is also a proteinaceous necrosis toxin and has a small molecular mass, ranging from 10 to 30 kDa (Abeysekara et al., 2009). As with most of these toxins, the necrosis symptoms are observed when the plants are illuminated (Abeysekara et al., 2009). *Snn4* is the host sensitivity gene whose protein product interacts with SnTox4 and results in necrosis. *Snn4* is located on the short arm of chromosome 1A (1AS) (Abeysekara et al., 2009).

SnTox5- *Snn5* interaction

The fifth proteinaceous necrosis toxin identified, SnTox 5, is also light dependent and ranges in size from 10 to 30 kDa (Friesen et al., 2012). The SnTox5 sensitivity gene, *Snn5*, is located on the long arm of chromosome 4B (4BL). SnTox5 works with SnToxA in an additive

manner, similar to that of SnToxA and SnTox2, where disease severity increases when SnToxA and SnTox5 are present along with their sensitivity genes *Snn5* and *Tsn1* (Friesen et al., 2012).

SnTox6- *Snn6* interaction

SnTox6 was recently identified as a small protein, about 12.3 kDa, which induces necrosis, and its activity is also light dependent, similar to most of the *P. nodorum* protein toxins (Gao et al., 2015). The *Snn6* gene confers sensitivity to SnTox6 and is located on the long arm of wheat chromosome 6A (6AL) (Gao et al., 2015).

SnTox7- *Snn7* interaction

The most recent toxin identified is SnTox7. The toxin has a mass of more than 30kDa.The *Snn7* gene confers sensitivity to SnTox7. The location of the gene is on wheat chromosome 2D, and *Snn7* is present in far fewer wheat cultivars compared to other sensitivity genes (Shi et al., 2015). SnToxA, produced by this pathogen, is identical to PtrToxA and was described with the other HSTs produced by *P. tritici-repentis*.

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CHAPTER 2: THE EFFECT OF CYTOPLASM SUBSTITUTION ON THE RESPONSE OF WHEAT TO HOST-SELECTIVE TOXINS PRODUCED BY FOLIAR PATHOGENS *P. TRITICI-REPENTIS* AND *P. NODORUM*

Abstract

The foliar necrotrophic pathogens *P. tritici-repentis* and *P. nodorum* are the causal agents of the foliar diseases tan spot and Stagonospora nodorum blotch on wheat, respectively. Both diseases can cause yield losses reaching 50%, under optimum conditions, and grain quality reductions. The two pathogens are known to produce HSTs that interact with proteins derived from host sensitivity genes, resulting in many of the disease symptoms. Lines of alloplasmic wheat have been shown to express altered responses to toxins from other pathogens, such as the *F. graminearum* toxin, deoxynivalenol, which causes Fusarium head blight in wheat (Wu et al., 1998). In this study, four HSTs, Ptr/SnToxA, Ptr ToxB, SnTox1, and SnTox3, from *P. tritici-repentis* and *P. nodorum* were used to determine their effect on alloplasmic lines derived from three parental lines, Selkirk, Chris, and durum wheat line 56-1. All alloplasmic lines responded to the toxins in a manner similar to the parental lines when they were infiltrated with the four toxins individually. Selkirk and its alloplasmic lines were insensitive when tested with Ptr/SnToxA, Ptr ToxB, and SnTox1. Chris and its alloplasmic lines showed a sensitive reaction to Ptr ToxB. Selkirk and Chris, and their alloplasmic lines, were sensitive to SnTox3. Durum wheat line 56-1 and its alloplasmic lines gave sensitive reactions to all toxins tested, except Ptr ToxB. This indicates that the responses, of the alloplasmic lines used in this study, to the toxins were controlled by nuclear genes and were not influenced by the substitution of the cytoplasmic genome.

Introduction

P. tritici-repentis and *P. nodorum* are necrotrophic pathogens that are the causal agents of the foliar diseases tan spot and Stagonospora nodorum blotch on wheat, respectively. Both fungi can cause yield and quality reductions by infecting the leaves and kernels. Under favorable environmental conditions, losses caused by these diseases can reach up to 50% during a single growing season (Eyal, 1981; Shabeer and Bockus, 1988). These pathogens are most often managed by crop rotation and tillage practices. Additionally, fungicides can be used to decrease disease severity in the field. However, the use of fungicides is costly and is not environmentally safe (Bockus and Claassen, 1992; Bockus and Shroyer, 1998; Eyal, 1999; Sutton and Vyn, 1990). These pathogens are known to produce multiple effectors, including toxins, which interact with host proteins produced from specific sensitivity genes in wheat, and results in susceptibility or disease. This process, where the interaction of two proteins results in disease rather than resistance, is known as the inverse gene-for-gene model. There are four known toxins produced by *P. tritici-repentis,* Ptr ToxA, Ptr ToxB, Ptr ToxC, and Ptr Nec, although not all isolates produce all toxins (Ali et al., 2010; Lamari et al., 1995; Orolaza et al., 1995; Tomás and Bockus, 1987). *P. nodorum* produces a larger number of toxins, including SnToxA, SnTox1, SnTox2, SnTox3, SnTox4, SnTox5, SnTox6, and SnTox7 (Abeysekara et al., 2009; Friesen et al., 2007, 2008, 2012; Gao et al., 2015; Liu et al., 2004; Shi et al., 2015). Developing wheat lines that are insensitive to these toxins can decrease the disease severity and is the most environmentally sound and least costly method.

The underlying mechanisms of some of these toxins have been determined. Toxins secreted by the pathogens may be localized in the apoplastic space or the cytoplasm of the host. In addition, in the cytoplasm these toxins may concentrate their effect on certain organelles, such

as the chloroplasts (Figueroa et al., 2015; Liu et al., 2012; Meinhardt et al., 2002). If a toxin targets an organelle, such as the mitochondria or chloroplast, then substitution of the cytoplasm in wheat cultivars, which will affect the interactions between the nucleus and the cytoplasm (alloplasmic wheat), may lead to changes in the response of the plants to the toxins, when compared to the original lines (euplasmic). For example, *Bipolaris maydis* race T, which is the casual pathogen of the disease Southern corn leaf blight, causes high disease severity only on hybrid maize carrying *cms-T* cytoplasmic sterility. Also, the pathogen *Phyllosticta maydis* affects maize with *cms-T* cytoplasm. *B. maydis* race T and *P. maydis* produce toxins, named BmT and Pm, which interact specifically with mitochondria of *cms-T* maize (Kono and Daly, 1979; Levings, 1990; Lim and Hooker, 1972). An additional example, *Fusarium graminearum*, which causes Fusarium head blight on wheat, also secretes toxins. The fungal toxin sensitivity was tested on inflorescence calli in an alloplasmic wheat line with *Ae. ventricosa* cytoplasm, and they displayed higher production of calli compared to the parental euplasmic wheat cultivar (Wu et al., 1998).

Alloplasmic lines of some crops may have altered disease sensitivity, either negatively or positively, and understanding the mechanisms behind these changes may help us in using them as new sources of resistance. Infiltration of culture filtrates and purified toxins into leaves can be helpful in studying the effects of toxins individually, and can show whether some alloplasmic lines may have changed in their sensitivity to the toxins. The intention of these experiments was to identify responses by alloplasmic lines of the wheat cultivars Chris, Selkirk, and 56-1 to four toxins produced by *P. nodorum* and *P. tritici-repentis* in comparison to the response of the parental lines.

Materials and methods

Plant Materials

In this study, 32 alloplasmic lines developed by Dr. S. S. Maan were used (Maan, 1975). Euplasmic and alloplasmic wheat lines (Table 2.1), were grown to the two to three leaf stage in the greenhouse, approximately 14 days after planting, in containers with two seeds per cone, and two cones per line. The containers were filled with Sunshine SB100 soil (Sun Grow Horticulture, Bellevue, WA), and fertilized with Osmocote Plus 15-19-12 fertilizer (Scotts Sierra Horticultural Product Company, Maysville, OH). Before infiltration, plants were placed under fluorescent lighting for 15 to 20 minutes to increase the ease of delivery of toxins through leaf stoma. When fully expanded, the second leaf was infiltrated with 25 µl of crude culture filtrate containing the toxin or the purified toxin using a 1 ml sterile needleless syringe, and the infiltrated area was marked using a nontoxic marker as previously described by Liu et al. (2004). Wheat differential lines were used as either positive or negative controls for each toxin: SnToxA/Ptr ToxA – Grandin (sensitive)/Chinese Spring (insensitive); SnTox1 – Chinese Spring (sensitive)/BR34 (insensitive); SnTox3 – Opata 85 (sensitive)/BR34 (insensitive); and Ptr ToxB – 6B662 (sensitive)/6B365 (insensitive). After infiltration, plants were kept in a growth chamber at 21°C and a 12 hour light/dark cycle. Responses were recorded 3 to 7 days after infiltration for necrosis or chlorosis in the infiltration area. The infiltration experiments were repeated three times to confirm the results.

Evaluation of HSTs

To test their sensitivity to toxins SnTox1, SnTox3, SnToxA, and Ptr ToxB, all lines were infiltrated with solutions containing each toxin. A control sample of Ptr ToxA, purified in our laboratory as previously described (Meinhardt et al., 2002), was used at a concentration of 10 µg/ml. Crude culture filtrate from *P. nodorum* isolate Sn79+SnTox3 was used as a source of SnTox3 for infiltrations as described previously (Liu et al., 2004). Both SnTox1 and Ptr ToxB were obtained as culture filtrates from *Pichia pastoris* expressing either SnTox1 or Ptr ToxB

(Abeysekara et al., 2009; Liu et al., 2012). *P. nodorum* isolate Sn79+SnTox3 and the *P. pastoris* lines expressing the toxins were a kind gift from Dr. Tim Friesen.

Table 2.1. Parental and alloplasmic wheat lines tested for toxin sensitivity.

Line	Cytoplasm Source	Nuclear Makeup	Nuclear Group	Generation
Grandin	*Triticum aestivum* Grandin	Grandin	*	*
Chris	*Triticum aestivum* Chris	Chris	1	*
SSM0191	*Aegilops crassa* Boiss.	Chris	4	selk8-chr2
SSM0192	*Aegilops crassa*	Chris	1	BC9
SSM0193	*Aegilops crassa*	Chris	1	BC3
SSM0194	*Aegilops cylindrica* Host	Chris	1	BC10
SSM0195	*Aegilops cylindrica*	Chris	1	BC14
SSM0196	*Aegilops juvenalis* (Thell.) Eig	Chris	1	BC15
SSM0197	*Aegilops kotschyi* Boiss.	Chris	4	selk13-chr3
SSM0198	*Aegilops longissima* Schweinf. & Muschl.	Chris	1	BC6
SSM0202	*Aegilops squarrosa* L.	Chris	1	BC18
SSM0206	*Aegilops variabilis* Eig	Chris	1	BC20
SSM0207	*Aegilops vavilovii* (Zhuk.) Chennav.	Chris	4	selk7-chr
SSM0208	*Aegilops ventricosa* Tausch	Chris	1	BC9
SSM0210	*Haynaldia triticum* D.	Chris	1	BC10
SSM0237	*Aegilops bicornis* (Forssk.) Jaub. & Spach	Chris	1	BC25
SSM0241	*Aegilops longissima*	Chris	1	BC5
SSM0242	*Aegilops mutica* Boiss.	Chris	1	BC9
SSM0254	*Triticum macha* 140191 Dekapr. & Menabde	Chris	1	BC21
Selkirk	*Triticum aestivum*	Selkirk	2	*
SSM0003	*Aegilops crassa* 6N	Selkirk	2	BC 7
SSM0004	*Aegilops cylindrica* Cw	Selkirk	2	Self at BC13
SSM0015	*Triticum dicoccoides* G1395 Schrank ex Schubi.	Selkirk	2	BC17
SSM0016	*Triticum dicoccoides* G1453	Selkirk	2	BC13
SSM0017	*Triticum dicoccoides* G1458	Selkirk	2	BC17
SSM0018	*Triticum dicoccoides* G1460	Selkirk	2	BC17
SSM0020	*Triticum dicoccoides* G803	Selkirk	2	BC13
SSM0022	*Triticum dicoccoides* Okla 11140	Selkirk	2	self at BC18
SSM0039	*Aegilops bicornis*	Selkirk	2	self at BC20
SSM0045	*Aegilops mutica*	Selkirk	2	BC11

[a] 1 = lines with Chris nucleus, 2 = lines with Selkirk nucleus, 3 = lines with 56-1 nucleus, 4 = lines with a mixed nucleus from Selkirk and Chris.
[b] BC = backcrossed, selk = Selkirk, chr = Chris, * = not backcrossed.

Results

Each of the nuclear donors (Chris, Selkirk, and 56-1) were tested for their sensitivity to Ptr ToxA (Fig. 2.1). Grandin was used as a positive control and showed the typical necrosis symptoms when injected with Ptr ToxA (Fig. 2.1A), while the water control (Fig. 2.1B) showed

only a small change due to physical damage to the leaf. Chinese Spring wheat was used as a negative control and gave the expected resistant response (Fig. 2.1C). Again, the water control showed no symptoms (Fig. 2.1D).

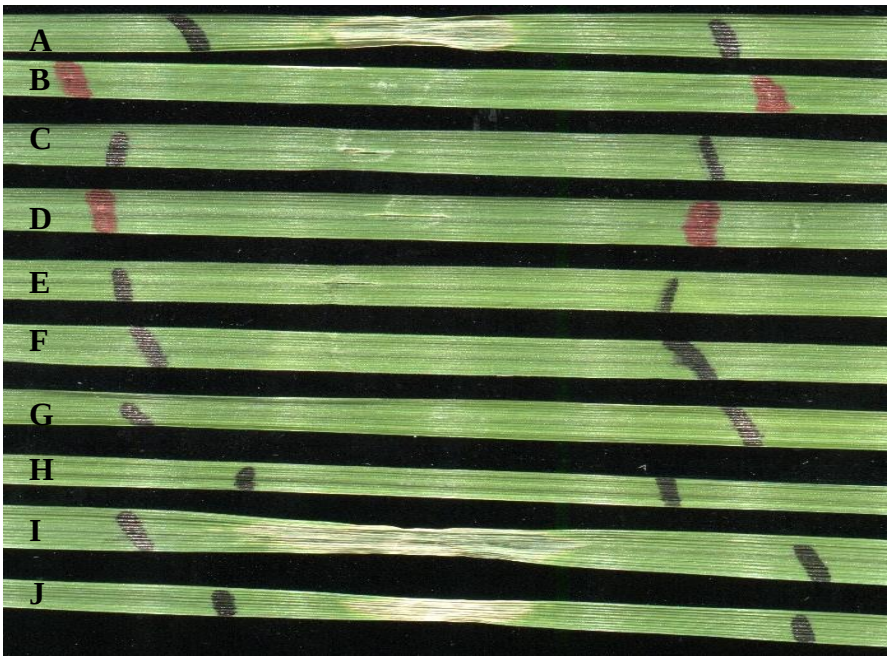

Figure 2.1. Reaction to Ptr ToxA for selected euplasmic and alloplasmic wheat lines. A & B Grandin (positive check and cont.), C & D Chinese Spring (negative and cont.), E & F Selkirk cv. and SSM0003 line (negative), G & H Chris cv. and SSM0195 line (negative), I & J 56-1 cv. and SSM0069 line (positive).

As expected, Selkirk was resistant to the toxin, as was previously reported (Lamari et al., 2005) (Fig. 2.1E). The alloplasmic line SSM0003 (Fig. 2.1F) also gave a resistant response, as did all of the alloplasmic lines with Selkirk as its nuclear donor. To our knowledge, there are no reports for toxin sensitivity for Chris or 56-1. Chris showed a resistant response (Fig. 2.1G), as did alloplasmic line SSM0195 (Fig. 2.1H). Again, all alloplasmic lines derived from Chris gave resistant responses. The durum line 56-1 gave a sensitive response to Ptr ToxA (Fig. 2.1I), as did its alloplasmic line SSM0069 (Fig. 2.1J). As with the other alloplasmic lines, all alloplasmic lines derived from 56-1 were sensitive to Ptr ToxA. In each case the water control showed little

or no change. For all 32 alloplasmic wheat lines tested for their sensitivity to Ptr ToxA, the results were similar to that obtained from their euplasmic parental line.

The results from testing the parental and alloplasmic lines for sensitivity to Ptr ToxB are given in Fig. 2.2. The positive control was line 6B662, where typical chlorosis was observed (Fig. 2.2A). The water control exhibited no symptoms (Fig. 2.2B). The line 6B365 was used as the negative control (Fig. 2.2C), along with a media control (Fig. 2.2D). Both showed no symptoms after infiltration. Selkirk gave a resistant response when infiltrated with ToxB, as has been previously reported (Lamari et al., 2005) (Fig. 2.2E), as did its alloplasmic line SSM0003 (Fig. 2.2F). Both Chris and its alloplasmic line SSM0195 were sensitive to ToxB (Fig. 2.2G and H). Lastly, line 56-1 and its alloplasmic lines were resistant to Tox B (Fig. 2.2I and J).

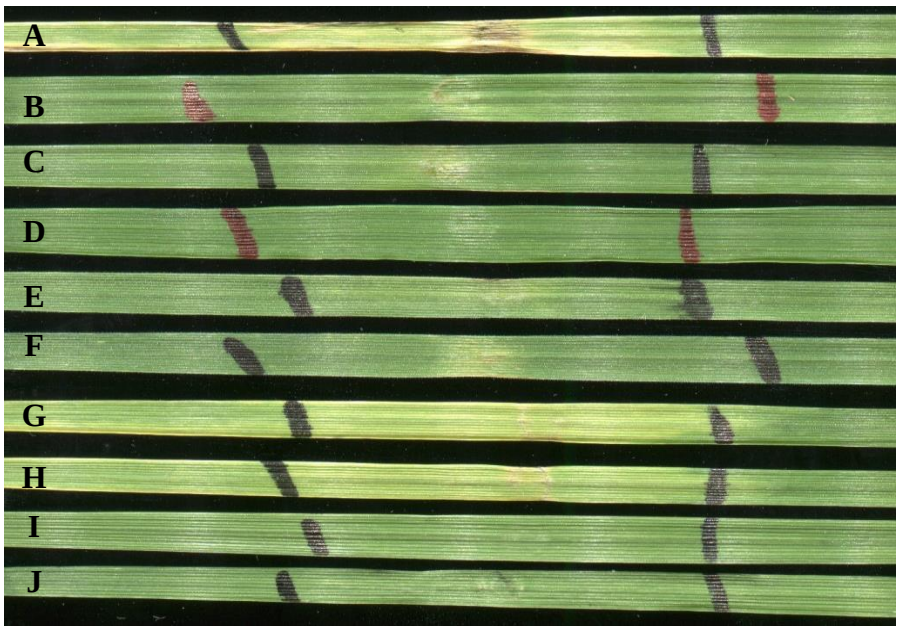

Figure 2.2. Reaction to Ptr ToxB for selected differentials, euplasmic, and alloplasmic wheat lines. A & B 6B662 (positive and cont.), C & D 6B365 (negative and cont.), E & F Selkirk cv. and SSM0003 line (negative), G & H Chris cv. and SSM0195 line (positive), I & J 56-1 cv. and SSM0069 line (negative).

The results from SnTox1 infiltrations of the euplasmic and alloplasmic lines are given in Fig. 2.3. Chinese Spring was used as a positive check and showed necrosis symptoms when

infiltrated with SnTox1 (Fig. 2.3A), but the media control (Fig. 2.3B) showed no symptoms. Line BR34 was used as negative check, and also showed no symptoms (Fig. 2.3C), and the media control (Fig. 2.3D) expressed no symptoms. Selkirk was also insensitive to the toxin (Fig. 2.3E). The alloplasmic line SSM0003 (Fig. 2.3F) expressed the same insensitivity to the toxin as the parental line and the rest of the Selkirk alloplasmic lines. Chris showed a resistant response to the toxin (Fig. 2.3G). The alloplasmic line SSM0195 showed a response similar to its parent Chris (Fig. 2.3H) as did all Chris alloplasmic lines. Durum wheat line 56-1 showed sensitivity to the toxin (Fig. 2.3I). Alloplasmic line SSM0069 (Fig. 2.3J) also showed sensitivity to the toxin as did all alloplasmic lines with parental line 56-1. All of the 32 alloplasmic lines tested for SnTox1 expressed responses similar to their parents.

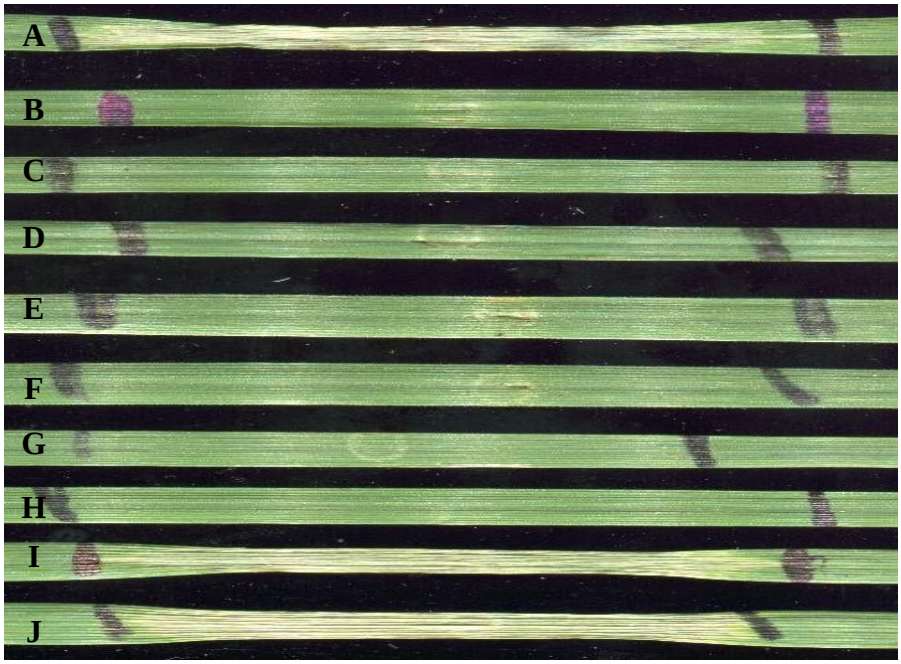

Figure 2.3. Reaction to SnTox1 for selected differentials, euplasmic, and alloplasmic wheat lines. A & B Chinese Spring (positive and cont.), C & D BR34 (negative and cont.), E & F Selkirk cv. and SSM0003 line (negative), G & H Chris cv. and SSM0195 line (negative), I & J 56-1 cv. and SSM0069 line (positive).

The results from testing the parental and alloplasmic lines for sensitivity to SnTox3 are given in Fig. 2.4. Opata 85 was used as the positive check and showed sensitivity to the toxin

when infiltrated (Fig. 2.4A), and insensitivity when treated with the media control (Fig. 2.4B). BR34 was used as negative check for the toxin, and showed insensitivity to it (Fig. 2.4C). When it was treated with media only it also showed no symptoms (Fig. 2.4D). Selkirk showed sensitivity to SnTox3 when infiltrated (Fig. 2.4E), in agreement with Shi et al. (2015). Selkirk alloplasmic line SSM0003 expressed symptoms similar to its parent (Fig. 2.4F). The rest of the Selkirk alloplasmic lines showed symptoms similar to the parent when infiltrated with the toxin. When Chris was infiltrated with SnTox3, it expressed sensitivity (Fig. 2.4G), as did the alloplasmic line SSM0195 (Fig. 2.4H). All Chris alloplasmic lines showed sensitivity to the toxin as well. The durum wheat 56-1 showed sensitivity to the toxin (Fig. 2.4I), as did its alloplasmic line SSM0069 (Fig. 2.4J). The rest of the 56-1 alloplasmic lines also expressed the same symptoms as the parental type. All alloplasmic lines tested for SnTox3 showed a sensitivity similar to their parental type to the toxin.

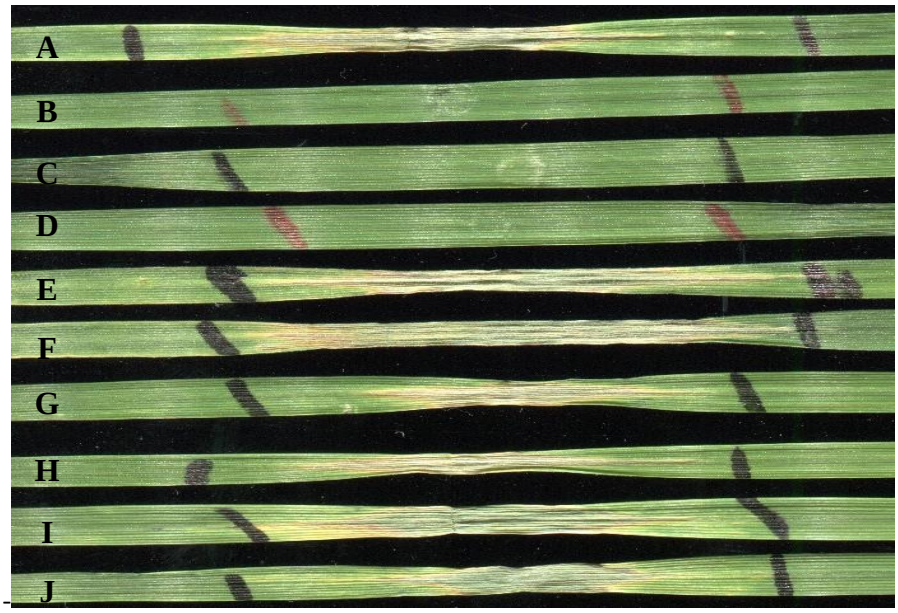

Figure 2.4. Reaction to SnTox3 for selected differentials, euplasmic, and alloplasmic wheat lines. A & B Opata 85 (positive and cont.), C & D BR34 (negative and cont.), E & F Selkirk cv. and SSM0003 line (positive), G & H Chris cv. and SSM0195 line (positive), I & J 56-1 cv. and SSM0069 line (positive).

Discussion

Infiltrations of four toxins produced by *P. nodorum* and *P. tritici-repentis* were used in phenotyping the alloplasmic lines, and their respective euplasmic parental lines: Chris, Selkirk, and 56-1. The alloplasmic progeny of these lines did not differ from their parental lines in their response to these toxins. Although the substitution of the cytoplasmic organelles, mitochondria and chloroplasts, from other grass species while maintaining the original nucleus may have resulted in changes in the phenotypes of the alloplasmic lines, this was not seen in the lines tested here. The response to all four of the toxins, SnTox1, SnTox3, Ptr ToxA, and Ptr ToxB, remained the same as the nuclear donor (Figs. 1, 2, 3, and 4). The toxin sensitivity of the alloplasmic lines of Chris, Selkirk, and 56-1 follows the inverse gene-for-gene model. The responses observed here were dependent on the nuclear genes of the parental lines, which contains the sensitivity genes for the toxins tested. In this project, only 32 alloplasmic wheat lines, derived from three cultivars and different cytoplasmic donors, were tested. There are other alloplasmic wheat lines that were not used in this study, which are derived from different nuclear and cytoplasmic donors, and may show changes in their responses to the HSTs (Maan, 1975; Panayotov, 1983; Suemoto, 1983; Tsunewaki et al., 1996; Washington and Maan, 1974; Wu et al., 1998). Since the responses of these alloplasmic lines to these toxins has followed the inverse gene-for-gene model, the increase in resistance of alloplasmic wheat lines may be due to other factors. Other toxins of *P. nodorum* and *P. tritici-repentis* will have to be tested on these lines to confirm which model these lines follow. If any of the other untested toxins follow a different model, it might be that there are other factors involved in increasing the resistance of alloplasmic wheat lines compared to their parents, and this will need to be identified in further studies. In conclusion, the alloplasmic condition of the tested lines did not affect the responses to the toxins

which were used in the infiltrations. All alloplasmic lines of Chris, Selkirk, and 56-1 maintained similar reactions to their parents, and their responses were based on sensitivity/insensitivity genes located in the nucleus.

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CHAPTER 3: THE EFFECT OF CYTOPLASM SUBSTITUTION ON THE RESPONSE OF WHEAT TO FOLIAR PATHOGENS *P. TRITICI-REPENTIS* AND *P. NODORUM* SPORE INOCULATIONS

Abstract

P. tritici-repentis and *P. nodorum* are foliar necrotrophic pathogens that are the causal agents of the foliar diseases tan spot and Stagonospora nodorum blotch on wheat, respectively. Both diseases can cause yield losses of up to 50% under optimum conditions, and cause quality reductions in the grain. Both can be controlled by using crop rotations, tillage practices, and fungicides, although the use of chemicals is not environmentally safe. Using resistant wheat varieties is the most effective means to control the diseases. One possible means of increasing the resistance of wheat to disease is to use alloplasmic derivatives. The substitution of the cytoplasmic genome, creating alloplasmic lines, may lead to changes in the interactions between the cytoplasmic and nuclear genomes, and can lead to changes in disease responses. Here, we investigate the responses of Chris, Selkirk, and 56-1 alloplasmic lines to infection with five different races of *P. tritici-repentis* (race 3/331-9, race 2/86-124, race 5/DW5, race 4/88-1, and ARcrossB10), and two isolates of *P. nodorum* (Sn2k and Sn4). In each case, the disease intensity was compared between the alloplasmic lines and their parental lines. All isolates used secrete different HSTs which results in differences in the disease levels observed. In most instances, no significant differences were observed between the alloplasmic lines and their parents. Only three alloplasmic lines showed significant increases in their disease resistance; SSM0039, SSM0237 and SSM0241. Line SSM0039 had increased resistance to one isolate of *P. nodorum*, Sn4, and one isolate of *P. tritici-repentis*, 331-9. Line SSM0241 had increased resistance to two *P. tritici-repentis* isolates, DW5 and 86-124. Line SSM0237 showed increased resistance to *P. tritici-*

repentis isolate 331-9. Two of these lines, SSM0039 and SSM0237, contained *Ae. bicornis* cytoplasm but different nuclear donors. The other two lines contained *Ae. longissima* cytoplasm and the Chris nucleus. The results suggest that *Ae. bicornis* and *Ae. longissima* may be good choices in making alloplasmic wheat lines that are fertile and have increased disease resistance.

Introduction

Common wheat is considered one of the most important crops globally. Wheat production is affected by biotic stresses that reduces the quality and quantity of the crop. *P. tritici-repentis* and *P. nodorum* are necrotrophic pathogens that cause the diseases tan spot and Stagonospora nodorum blotch on wheat, respectively. Both pathogens can cause up to 50% yield losses (Eyal, 1981; Shabeer and Bockus, 1988). Management of both diseases can be accomplished by applying crop rotation and tillage practices. Fungicide use is also effective, but it is costly and considered environmentally unsound. Providing resistant varieties against both pathogens is the most effective and environmentally safe means of protecting the crop (Bockus and Claassen, 1992; Bockus and Shroyer, 1998; Eyal, 1999a; Sutton and Vyn, 1990). Both pathogens are able to produce host-specific toxins (HSTs) that interact with host specific proteins produced from dominant susceptibility genes. This differs from biotrophic pathogens, where interactions between the effectors and the proteins encoded by other dominant genes results in resistance. Knowing and understanding the mechanisms behind these interactions may help in developing wheat varieties that have resistance against these pathogens. Researchers and breeders have been identifying resistance genes and developing resistant wheat varieties against both pathogens. Many nuclear resistance genes have been identified from grasses related to wheat and introduced into common wheat by crossing. For example, some *Aegilops* spp. have been used as source for resistance genes against these pathogens as well as others (Friesen et al.,

2008; Jones et al., 1995). Another, as yet untapped, possibility is the use of the genomes found in the cytoplasm, in both mitochondria and chloroplasts, from related grasses. The interactions between the cytoplasmic and nuclear genomes may have an effect on disease susceptibility, resulting in either increased resistance or susceptibility against several wheat pathogens. For instance, alloplasmic wheat lines of Chris cv. with an alien cytoplasm from *Aegilops* spp. showed increased resistance against *P. nodorum* compared with parental line Chris (Keane and Jones, 1990). The intention of this project was to evaluate the effect of the substitution of the cytoplasm in wheat with cytoplasms from related grasses in an effort to find increased resistance or susceptibility to the two foliar pathogens, *P. nodorum* and *P. tritici-repentis.*

Materials and methods

Plant Materials

In this study, 32 alloplasmic lines developed by Dr. S. S. Maan were used (1975). Selection of these lines was based on the presence of fertility, and, in most cases, having been back-crossed at least 10 times, to insure they possessed a nucleus identical to the parental line. The alloplasmic lines had one of three possible nuclear donors, the bread wheat cultivars Selkirk or Chris, or the durum wheat line 56-1. The lines used are listed in Table 3.1. All lines were planted in cone-tainers filled with Sunshine SB100 soil (Sun Grow Horticulture, Bellevue, WA), and fertilized with Osmocote Plus 15-19-12 fertilizer (Scotts Sierra Horticultural Product Company, Maysville, OH). For experiments with *P. nodorum*, two seeds were planted in each cone, and each experiment was replicated six times. Cone-tainers were arranged in 98 cone racks in a complete randomized design (CRD).

Table 3.1. Parental and alloplasmic wheat lines tested for spore inoculations.

Line	Cytoplasm Source	Nuclear Makeup	Nuclear Group[a]	Generation[b]
Grandin	*Triticum aestivum* Grandin	Grandin	*	*
Chris	*Triticum aestivum* Chris	Chris	1	*
SSM0191	*Aegilops crassa*	Chris	4	selk8-chr2
SSM0192	*Aegilops crassa*	Chris	1	BC9
SSM0193	*Aegilops crassa*	Chris	1	BC3
SSM0194	*Aegilops cylindrica*	Chris	1	BC10
SSM0195	*Aegilops cylindrica*	Chris	1	BC14
SSM0196	*Aegilops juvenalis*	Chris	1	BC15
SSM0197	*Aegilops kotschyi*	Chris	4	selk13-chr3
SSM0198	*Aegilops longissima*	Chris	1	BC6
SSM0202	*Aegilops squarrosa*	Chris	1	BC18
SSM0206	*Aegilops variabilis*	Chris	1	BC20
SSM0207	*Aegilops vavilovii*	Chris	4	selk7-chr
SSM0208	*Aegilops ventricosa*	Chris	1	BC9
SSM0210	*Haynaldia triticum* D.	Chris	1	BC10
SSM0237	*Aegilops bicornis*	Chris	1	BC25
SSM0241	*Aegilops longissima*	Chris	1	BC5
SSM0242	*Aegilops mutica*	Chris	1	BC9
SSM0254	*Triticum macha* 140191	Chris	1	BC21
Selkirk	*Triticum aestivum*	Selkirk	2	*
SSM0003	*Aegilops crassa* 6N	Selkirk	2	BC 7
SSM0004	*Aegilops cylindrica* Cw	Selkirk	2	Self at BC13
SSM0015	*Triticum dicoccoides* G1395	Selkirk	2	BC17
SSM0016	*Triticum dicoccoides* G1453	Selkirk	2	BC13
SSM0017	*Triticum dicoccoides* G1458	Selkirk	2	BC17
SSM0018	*Triticum dicoccoides* G1460	Selkirk	2	BC17
SSM0020	*Triticum dicoccoides* G803	Selkirk	2	BC13
SSM0022	*Triticum dicoccoides* Okla 11140	Selkirk	2	self at BC18
SSM0039	*Aegilops bicornis*	Selkirk	2	self at BC20
SSM0045	*Aegilops mutica*	Selkirk	2	BC11
SSM0054	*Aegilops crassa*	Selkirk	2	BC12
56-1	*Triticum durum* 56-1	56-1	3	*
SSM0069	*Aegilops longissima khaplis*	56-1	3	Self3-BC2
SSM0085	*Aegilops variabilis*	56-1	3	Self2-op@BC10
SSM0076	*Aegilops sharonensis*	56-1	3	Self2-BC2
SSM0107	*Triticum dicoccoides* G1453	56-1	3	Self2-BC15

[a] 1 = lines with Chris nucleus, 2 = lines with Selkirk nucleus, 3 = lines with 56-1 nucleus, 4 = lines with a mixed nucleus from Selkirk and Chris.
[b] BC = backcrossed, selk = Selkirk, chr = Chris, * = not backcrossed.

In the experiments with *P. tritici-repentis*, plants were grown as above, except three seeds were planted per cone, each genotype had two replicates per experiment, and were arranged in the cone-container rack in a randomized complete block design (RCBD). Grandin and BR34 were used as susceptible and resistant checks, respectively, for *P. nodorum*. Grandin and Jerry were

used as susceptible checks, and the tan spot differential lines Salamouni, Glenlea, 6B662, and 6B365, were used to indicate toxin production. Plants were grown in the greenhouse at temperatures between 20° and 25°C and were inoculated when they reached the two to three leaf stage, approximately 14 days after planting.

Fungal Inoculations and Disease Evaluation for *P. tritici-repentis*

Five isolates of *P. tritici-repentis* were used in this study, each belonging to a different race; 86-124 (race 2), 331-9 (race 3), 88-1 (race 4), DW5 (race 5), and ARcrossB10 (race not determined). Race 2 isolates produce Ptr ToxA, race 3 isolates produce Ptr ToxC, race 5 isolates produce Ptr ToxB, and race 4 isolates produce no toxins (Table 3.2). Isolate ARcrossB10 is known to produce Ptr ToxC, but does not fit in with the current classification system because it produces necrosis on wheat line Glenlea, but does not possess the gene for ToxA (Ali et al., 2010). Although these isolates have been shown to express these toxins, additional toxins may be produced during infection.

Table 3.2. Host-selective toxins produced by isolates and races of *P. nodorum* and *P. tritici-repentis* used in this study.

Pathogen	Isolate	Toxins	Race[a]	References
P. tritici-repentis	86-124	Ptr ToxA	2	Ali and Francl, 2001
	331-9	Ptr ToxC	3	Ali and Francl, 2001
	88-1	None	4	Ali and Francl, 2001
	DW5	Ptr ToxB	5	Ali and Francl, 2001
	ARcrossB10	Ptr Nec, Ptr ToxC	*	Ali and Francl, 2001; Ali et al., 2010
P. nodorum	Sn2k	SnTox1, SnTox5, SnToxA	*	Liu et al., 2009; Friesen et al., 2012
	Sn4	SnTox1, SnTox2, SnTox3, SnToxA	*	Liu et al., 2009

[a] * = no race.

Inoculum preparation was based on the method described by Lamari and Bernier (1989) for conidia production. All isolates were grown on V8–potato dextrose agar for approximately 5

days. The fungal growth on the plates was flooded with sterilized distilled water and flattened using the bottom of a flame sterilized test tube. The plates were then placed in the light at room temperature for 24 hours, followed by moving to an incubator for 24 h in the dark at 16ºC for sporulation. The conidia were harvested by flooding the plate with sterilized distilled water, and gently scraping the spores using a sterilized inoculating loop. The liquid containing the spores was poured through two layers of cheesecloth into a beaker containing a stir bar for mixing. The spore concentration was adjusted to 650 spores/ml and two drops of Tween-20 were added per 100 ml. This spore concentration was chosen to allow measurement of the disease severity for 7 days after inoculation.

Plants were inoculated using a paint sprayer that was attached to a compressed air source at an air pressure of 1.0 bar. Plants were sprayed until runoff from the leaves. The plants were placed in a mist chamber (100% relative humidity) at room temperature for 24 h with 20 seconds misting time every four minutes under fluorescent light. After inoculation, the plants were kept in an incubator at 21ºC with a 12 h photoperiod for 7-10 days, and then scored.

The scale used for measuring the disease severity was based on lesion type, as described by Lamari and Bernier (1989), where 1 = small dark-brown to black spots without chlorotic borders (highly resistant); 2 = small dark-brown to black spots surrounded by little chlorosis or necrosis (moderately resistant); 3 = small dark-brown to black spots surrounded by distinct chlorosis or necrosis, but not merging (moderately susceptible); 4 = small dark-brown to black spots surrounded by distinct chlorosis or necrosis merged together (susceptible); and 5 = dark-brown or black spots with centers expanded, most lesions are merged with each other with severe chlorosis and necrosis throughout the leaves (highly susceptible).

Fungal Inoculations and Disease Evaluation for *P. nodorum*

Two isolates of *P. nodorum* were used; Sn2k, which produces SnToxA, SnTox1, and SnTox5; and Sn4, which produces SnToxA, SnTox1, SnTox2, and SnTox3. Inoculum preparation was based on the method described by Liu et al. (2004). Petri dishes containing V8–potato dextrose agar were inoculated, and the fungus was grown for 7 days under continuous light at room temperature, in order to obtain a high number of mature pycnidia. The spores were then harvested by adding 20 ml of sterilized distilled water to the plate, and the surface was scraped using a sterilized inoculation loop. The spore suspension was adjusted to 1×10^6 conidia per ml for inoculation. Two drops of Tween-20 were added per 100 ml of the final volume. The plants were inoculated and treated as described above for *P. tritici-repentis* inoculations.

Only the second leaf of the tested lines was evaluated for disease levels. A qualitative scale was used for measuring disease severity at 5, 7, and 10 days post inoculation, and was based on the scale described by Liu et al. (2004). The scale ranges from 0 to 5; 0 = no lesion observed (highly resistant); 1 = penetration points with small dark spots (resistant); 2 = dark spots surrounded by little necrosis or chlorosis (moderately resistant); 3 = dark spots surrounded completely by necrosis or chlorosis (moderately susceptible); 4 = large necrotic or chlorotic lesions but not merged together (susceptible); and 5 = necrosis or chlorosis merged and covers most of the leaf area (highly susceptible).

Statistical Analysis

Differences between lines, races, and their interactions were analyzed in SAS 9.4 using a procedure based on a generalized linear model (GLM) method. For analysis, data from all alloplasmic lines, with the same nuclear donor and inoculated with the same isolate, were

combined. Fisher's LSD test was used for means separation. Statistical differences among the different treatments was estimated at 95% confidence level.

Results

The data from three independent experiments were combined for each parental group for each isolate and analyzed. Based on the parental type there were four different alloplasmic groups: fourteen Chris alloplasmic lines, eleven Selkirk alloplasmic lines, four 56-1 (durum) alloplasmic lines, and three alloplasmic lines with a mixed genetic background of both Chris and Selkirk. Significant differences were observed between line, race/isolate, and line*race/isolate at 7 days after inoculation (DAI) with the two isolates of *P. nodorum* and the five races of *P. tritici-repentis*. Because the interaction between line*race was significant, no statistical comparison was made between the results for each isolate or race. Significant differences were calculated from comparing the alloplasmic lines to their respective euplasmic lines within each isolate or race only. Ratings for all lines were put into categories based on their average means. For *P. nodorum*, an average from 0 to 1 was highly resistant to resistant; 1 to 2 was resistant to moderately resistant; 2 to 3 was moderately resistant to moderately susceptible; 3 to 4 was moderately susceptible to susceptible; and from 4 to 5 was susceptible to highly susceptible. Categories used for *P. tritici-repentis* were 1 to 2 highly resistant to moderately resistant; from 2 to 3 moderately resistant to moderately susceptible; from 3 to 4 moderately susceptible to susceptible; and from 4 to 5 susceptible to highly susceptible.

Evaluation of the disease resistance/susceptibility of the euplasmic and alloplasmic lines to *P. nodorum* was conducted with two isolates (Tables 3.3 and 3.4). These two isolates gave different levels of disease on the lines tested. Grandin, which is the highly susceptible control, was used to determine the efficacy of the inoculum, and with both isolates gave a disease rating

of 5. In general, isolate Sn2k appears to be less aggressive than Sn4 on the alloplasmic lines tested in this study. However, when Sn2k was inoculated on the durum 56-1 alloplasmic lines, it showed a higher disease intensity compared to Sn4 (Figs. 3.1 and 3.2). These two isolates are known for producing different HSTs, and that may explain some of the disease variability observed (Table 3.3).

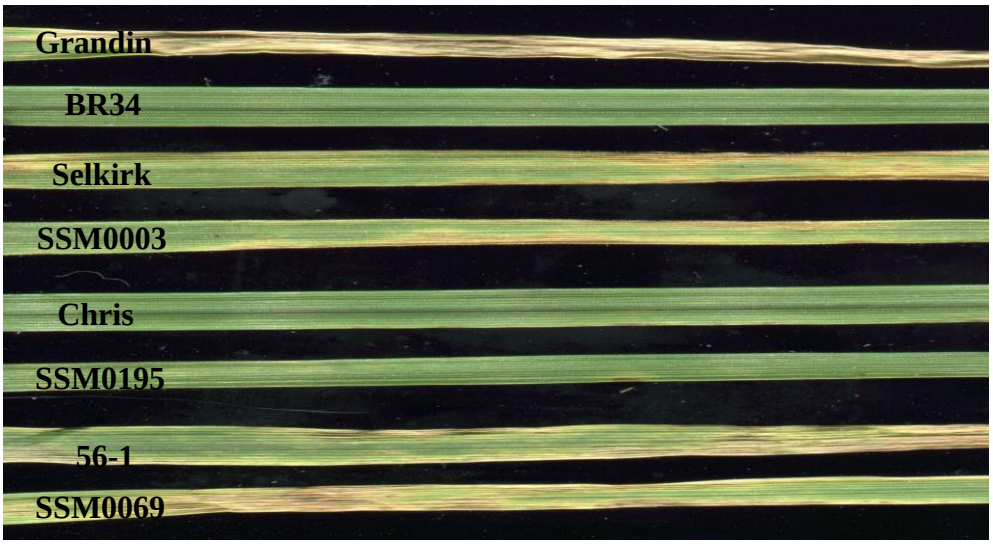

Figure 3.1. Reaction of resistant and susceptible checks, parental lines, and selected alloplasmic lines to *P. nodorum* isolate Sn2k.

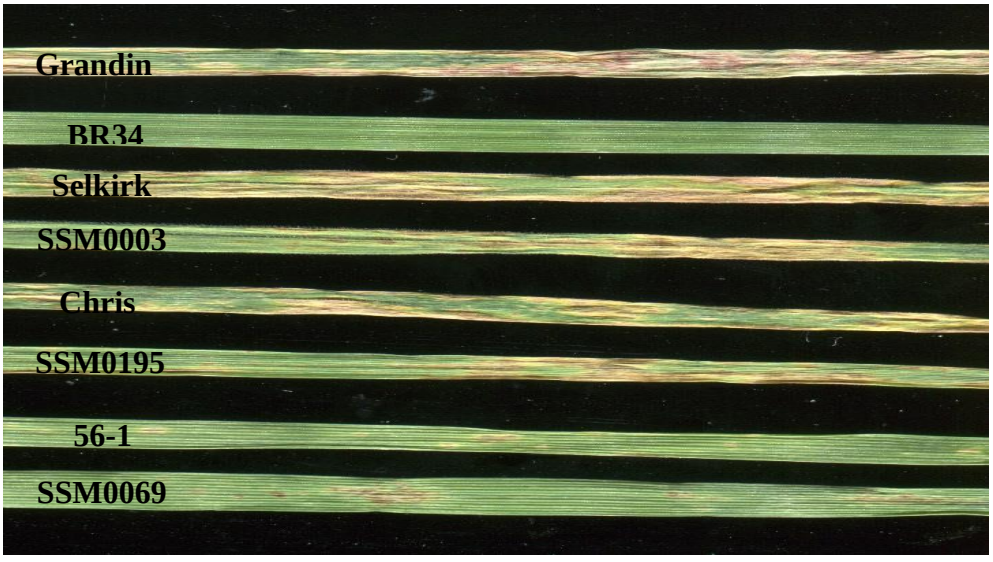

Figure 3.2. Reaction of resistant and susceptible checks, parental lines, and selected alloplasmic lines to *P. nodorum* isolate Sn4.

Chris and the fourteen alloplasmic lines derived from Chris, were inoculated with both *P. nodorum* isolates. The disease readings from both isolates produced no significant differences, when comparing the alloplasmic lines with their parental line, Chris, at 7 DAI, based on the calculated confidence intervals (Table 3.3). However, some lines showed a lower or higher average disease rating than the parental line Chris. Chris showed a high level of disease resistance to Sn2k, with a rating of 0.6, and a high level of susceptibility to Sn4, with a rating of 4.3 (Fig. 3.1).

Chris alloplasmic lines infected with Sn2k gave disease ratings between 0.5-2, indicating they were highly resistant to resistant, as was their nuclear parent. Eleven out of fourteen Chris alloplasmic lines evaluated with Sn2k had higher average disease rating scores: SSM0192, SSM0193, SSM0194, SSM0195, SSM0196, SSM0202, SSM0206, SSM0208, SSM0210, SSM0237, and SSM0254. Two of the other lines, SSM0198 and SSM0241, exhibited less disease intensity based on ratings. Sn4 developed a high level of disease intensity on the Chris alloplasmic lines, ranging from moderately susceptible to highly susceptible, again following the parental line Chris. When infected with Sn4, only five alloplasmic lines: SSM0192, SSM0196, SSM0202, SSM0210, and SSM0254, had higher average disease ratings when compared to Chris. The other alloplasmic lines: SSM0193, SSM0194, SSM0195, SSM0198, SSM0208, SSM0237, SSM0241, and SSM0242, had lower average disease scores compared to the parental line Chris (Table 3.3).

Table 3.3. Average disease scores of Chris, Selkirk, and 56-1 alloplasmic wheat lines, and confidence intervals for *P. nodorum* isolates Sn2k and Sn4.

Line	Cytoplasm Name	Nuclear Makeup	Mean		Lower and Upper Confidence Intervals	
			Sn2k	Sn4	Sn2k	Sn4
Chris	*T. aestivum*	Chris	0.6250	4.3056	0.1739-1.0761	3.9629-4.6482
SSM0192	*Ae. crassa*	Chris	0.6944	4.4722	0.3853-1.0036	4.1834-1.7611
SSM0193	*Ae. crassa*	Chris	1.5833	4.3056	1.0356-2.131	3.9737-4.6374
SSM0194	*Ae. cylindrica*	Chris	0.6389	4.1389	0.3220-0.9558	3.8107-4.4671
SSM0195	*Ae. cylindrica*	Chris	1	4.2778	0.5822-1.4178	3.9921-4.5635
SSM0196	*Ae. juvenalis*	Chris	0.8333	4.4167	0.4425-1.2242	4.0523-4.781
SSM0198	*Ae. longissima*	Chris	0.3333	3.5	0.5240-0.1426	2.8071-4.1929
SSM0202	*Ae. squarrosa*	Chris	1.2778	4.3333	0.8089-1.7466	3.9243-4.7423
SSM0206	*Ae. variabilis*	Chris	0.9167	4.5	0.5523-1.281	4.2171-4.7829
SSM0208	*Ae. ventricosa*	Chris	1.1389	4.1944	0.6430-1.6348	3.7584-4.6305
SSM0210	*Haynaldia triticum* D.	Chris	1.2222	4.3333	0.7534-1.6911	4.0921-4.5746
SSM0237	*Ae. bicornis*	Chris	0.8889	3.4444	0.2946-1.4832	2.8925-3.9964
SSM0241	*Ae. longissima*	Chris	0.2778	3.7778	0.0650-0.4905	3.3328-4.2228
SSM0242	*Ae. mutica*	Chris	0.7143	3.7	-0.2796-1.7081	2.9237-4.4763
SSM0254	*T. macha* 140191	Chris	1.5	4.6667	0.7763-2.2237	4.4578-4.8756
Selkirk	*T. aestivum*	Selkirk	1.8611	4.7222	1.0351-2.8712	4.5273-4.9171
SSM0003	*Ae. crassa* 6N	Selkirk	1.8056	4.4722	1.4134-2.1976	4.1368-4.8077
SSM0004	*Ae. cylindrica* Cw	Selkirk	1.3333	4.4722	0.6727-1.9939	4.2391-4.7054
SSM0015	*T. dicoccoides* G1395	Selkirk	1.5278	4.6944	0.7393-2.3162	4.4831-4.9058
SSM0016	*T. dicoccoides* G1453	Selkirk	1.7778	4.5556	1.0580-2.4975	4.2874-4.8238
SSM0017	*T. dicoccoides* G1458	Selkirk	1.6944	4.6389	1.0009-2.3880	4.4006-4.8772
SSM0018	*T. dicoccoides* G1460	Selkirk	1.8056	4.5278	0.9105-2.7006	4.1514-4.9041
SSM0020	*T. dicoccoides* G803	Selkirk	2.3333	4.25	1.4553-3.2114	3.9062-4.5938
SSM0022	*T. dicoccoides* Okla 11140	Selkirk	1.5	4.1667	0.7516-2.2484	3.7318-4.6015
SSM0039	*Ae. bicornis*	Selkirk	1.2941	3.9444	0.7117-1.8765	3.5546-4.3342
SSM0045	*Ae. mutica*	Selkirk	1.3611	4.4444	0.6746-2.0476	3.9860-4.9028
SSM0054	*Ae. crassa*	Selkirk	1.3611	4.5	0.6384-2.0838	4.1809-4.8191

Table 3.3. Average disease scores of Chris, Selkirk, and 56-1 alloplasmic wheat lines, and confidence intervals for *P. nodorum* isolates Sn2k and Sn4 (continued).

Line	Cytoplasm Name	Nuclear Makeup	Mean		Lower and Upper Confidence Intervals	
			Sn2k	Sn4	Sn2k	Sn4
56-1	*T. durum* 56-1	56-1	4.6667	2.3056	4.3476-4.9857	1.4564-3.1547
SSM0069	*Ae. longissima khaplis*	56-1	4.5278	1.8	4.0493-5.0062	1.0043-2.5957
SSM0076	*Ae. sharonensis*	56-1	4.8611	3.0278	4.7183-5.0039	2.2820-3.7736
SSM0085	*Ae. variabilis*	56-1	4.8056	1.8611	4.6545-4.9566	1.3341-2.5889
SSM0107	*T. dicoccoides* G1453	56-1	4.6667	1.1176	4.2577-5.0757	0.4780-1.7573

Eleven Selkirk alloplasmic lines were evaluated with the two isolates of *P. nodorum*. The average disease score rating for the Selkirk derived alloplasmic lines with Sn2k was 1.86 (Table 3.3). With Sn4, the average disease score rating was 4.72. Selkirk alloplasmic wheat lines gave a resistant reaction, with values falling between 1 and 2, with Sn2k (Fig. 3.1). With Sn4, a highly susceptible reaction was observed, with values between 4 and 5 (Fig. 3.2). None of the Selkirk alloplasmic lines inoculated with Sn4 showed any significant difference from its parent, except for line SSM0039 (Fig. 3.3).

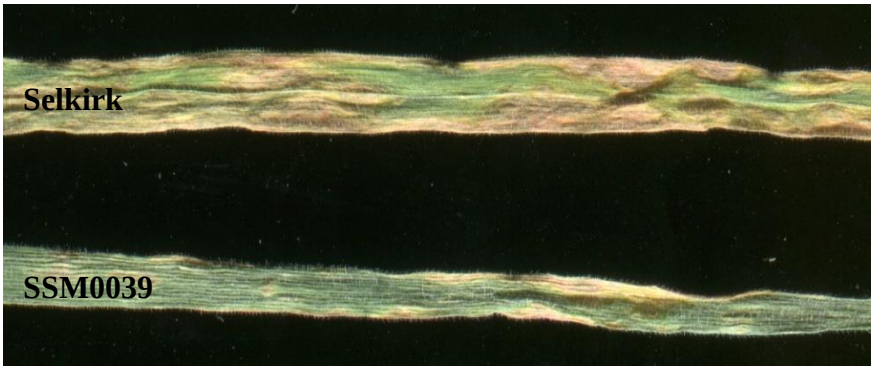

Figure 3.3. Reaction of Selkirk and SSM0039 to *P. nodorum* isolate Sn4, which were significantly different.

The mean disease score of SSM0039 with Sn4 was 3.94, which falls into the susceptible rather than highly susceptible category on the disease scale. The other lines had lower average

disease score ratings, but were not significantly different: SSM0003, SSM0004, SSM0015, SSM0016, SSM0017, SSM0018, SSM0020, SSM0022, SSM0045, and SSM0054. When inoculated with Sn2k, none of the alloplasmic lines showed significant differences when compared to Selkirk. Lines SSM0003, SSM0004, SSM0015, SSM0016, SSM0017, SSM0018, SSM0022, SSM0039, SSM0045, and SSM0054, showed lower average disease score ratings than Selkirk. For the line SSM0020, a higher average disease rating was obtained, but it was not statistically different from the parent Selkirk.

The third parental group consisted of the four 56-1 durum alloplasmic wheat lines (Fig. 3.1). The evaluation of the disease resistance or susceptibility for these lines with both isolates showed no significant differences between the parental line and its alloplasmic lines. When inoculated with Sn2k, the average disease rating score was 4.67 for 56-1, and its alloplasmic lines had readings that fell between 4.5 to 4.8, which are almost highly susceptible (Table 3.3). Two alloplasmic lines, SSM0069 and SSM0107, showed less disease, and the other two, SSM0076 and SSM0085, showed higher disease, when compared to the parent. As mentioned before, Sn2k was not highly aggressive with the other parental groups, but was highly aggressive with the 56-1 alloplasmic lines. Conversely, Sn4 was less aggressive with 56-1 and its alloplasmic lines, but was very aggressive with the other parental groups (Figs 3.1 and 3.2). With Sn4, the readings of these lines fell between 1 and 3, giving a resistant to moderately susceptible reaction. The parent, 56-1, had a score of 2.3. Three of the alloplasmic lines showed a lower average disease rating, SSM0069, SSM0085, and SSM0107, and one, SSM0076, gave a higher average disease score (Table 3.3).

The last group of alloplasmic lines has a mixture of nuclear genes derived from both Selkirk and Chris. The group was evaluated with the same two isolates from *P. nodorum*. In this

group the three alloplasmic wheat lines were compared with the two parental lines, Selkirk and Chris, for changes in their resistance (Table 3.4). When these lines were inoculated with Sn2k, they exhibited highly resistant to resistant reactions, with responses falling between 0.7 and 2. The parent Chris had an average disease rating of 0.6, and Selkirk's rating was 1.9.

Evaluation of the lines inoculated with Sn4 gave values ranging from 4.3 to 4.7, which are almost highly susceptible. Chris and Selkirk had average disease scores of 4.3 and 4.72, respectively. There were no significant differences when these lines were inoculated with Sn4 and compared to both parental lines. However, based on the average score three lines SSM0191, SSM0197, and SSM0207, had average disease scores higher than Chris. When compared to Selkirk, the only line with a higher average disease score was SSM0207, while the other two were less. When evaluated for disease caused by Sn2k, these lines showed a significant difference, however, only when compared to one parent. The average disease scores for Chris and Selkirk were 0.62 and 1.86, respectively. Line SSM0197 had an average score of 0.72, which was significantly different compared to Selkirk, but not to Chris (Fig. 3.4). Also, SSM0207 and SSM0191 had significantly higher average disease scores when compared with Chris only (Table 3.4).

Table 3.4. Average disease scores of alloplasmic wheat lines with a mixed nuclear background of Chris and Selkirk, and confidence intervals for *P. nodorum* isolates Sn2k and Sn4.

Line	**Cytoplasm Name**	**Nuclear Makeup**	**Mean**		**Lower and Upper Confidence Intervals**	
			Sn2k	**Sn4**	**Sn2k**	**Sn4**
Chris	*T. aestivum*	Chris	0.625	4.3056	0.1739-1.0761	3.9629-4.6481
Selkirk	*T. aestivum*	Selkirk	1.8611	4.7222	1.0351-2.6871	4.5273-4.9171
SSM0191	*Ae. crassa*	Self2-selk8-chr2	2.0833	4.3235	1.3534-2.8132	4.0095-4.6346
SSM0197	*Ae. kotschyi*	Self2-selk13-chr3	0.7222	4.4722	0.4495-0.9949	4.1261-4.8184
SSM0207	*Ae. vavilovii*	Self2-selk7-chr	1.6944	4.75	1.1747-2.2142	4.5307-4.9693

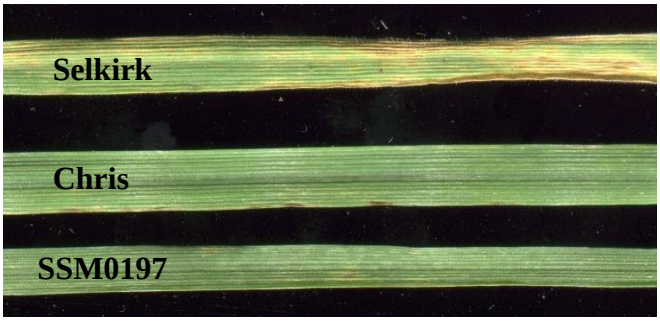

Figure 3.4. Reaction of Selkirk, Chris, and SSM0197 to *P. nodorum* isolate Sn2k, which were significantly different.

Evaluation of disease susceptibility/resistance of the alloplasmic lines to *P. tritici-repentis* was conducted with five different races (Table 3.5). These five races secrete different HSTs and variability of disease ratings were observed on the lines tested (Table 3.5). Grandin was used as a susceptible control, and showed a highly susceptible reaction to these races, by day 7. The line Chris showed a variable disease response when tested with the five races (Table 3.5).

With the race 3 isolate, 331-9, Chris gave an average disease score of 3.47, which is considered moderately susceptible (Fig. 3.5). When the Chris alloplasmic lines were tested with this race, two alloplasmic lines had a significant difference in disease ratings, compared to the parental line Chris (Table 3.5). Line SSM0237 had a significantly lower disease score compared to Chris, with an average disease score of 1.72 (Fig. 3.6). With isolate 331-9, the other alloplasmic lines had higher or lower average disease scores, but were not significantly different from the parental line Chris.

Table 3.5. Average disease scores of Chris, Selkirk, and 56-1 alloplasmic wheat lines to five *P. tritici-repentis* isolates/races; 331-9 (race 3), 86-124 (race 2), DW5 (race 5), ARcrossB10 (race not determined), and 88-1 (race 4).

Line	**Cytoplasm Name**	**Nuclear Makeup**	**Mean Lower and Upper Confidence Intervals**				
			331-9	**86-124**	**DW5**	**ARcrossB10**	**88-1**
Chris	*T. aestivum*	Chris	3.4722 (2.4926-4.4518)	4.4445 (3.5145-5.3744)	4.5556 (4.1943-4.9168)	4.6111 (4.0507-5.1715)	1.3888 (0.8285-1.9793)
SSM0192	*Ae. crassa*	Chris	3.8889 (2.6637-5.1141)	4.6388 (4.0492-5.2286)	4.7222 (4.1618-4.9168)	4.9445 (4.8016-5.0873)	1.7778 (0.8479-2.7077)
SSM0193	*Ae. crassa*	Chris	3.5278 (2.8245-4.2310)	4.8888 (4.60337-5.1745)	4.5556 (3.9430-5.1681)	4.6667 (4.0312-5.3021)	2.25 (1.6267-2.8733)
SSM0194	*Ae. cylindrica*	Chris	3.7223 (3.1197-4.3247)	4.5556 (3.9042-5.2069)	4.8333 (4.4049-5.2618)	4.8333 (4.5407-5.1260)	1.8322 (1.304-2.3604)
SSM0195	*Ae. cylindrica*	Chris	3.4999 (2.8841-4.1159)	4.7778 (4.4922-5.0634)	4.2777 (3.6753-4.8803)	4.5555 (3.8677-5.2434)	1.6667 (0.967-2.36639)
SSM0196	*Ae. juvenalis*	Chris	3.1944 (2.3205-4.0684)	4.1945 (3.2025-5.1864)	4.6667 (4.3534-4.9796)	4.0833 (3.1893-4.8477)	1.4445 (0.6887-2.20013)
SSM0198	*Ae. longissima*	Chris	1.8055 (1.0618-3.5493)	2.8611 (1.7307-3.9915)	3.6111 (2.593-4.6290)	3.8888 (2.6439-5.1339)	1.2222 (0.7443-1.7002)
SSM0202	*Ae. squarrosa*	Chris	3.6945 (2.4215-4.9674)	3.8195 (3.2330-4.4059)	4.6667 (4.2242-5.1092)	4.4445 (3.6570-5.2319)	1.3889 (0.6414-2.1364)
SSM0206	*Ae. variabilis*	Chris	2.3055 (1.0618-3.5493)	3.8889 (2.9078-4.8700)	4.3333 (3.5055-5.1611)	4.6945 (4.2744-5.1145)	1.4167 (0.5907-2.2426)
SSM0208	*Ae. ventricosa*	Chris	3.9445 (3.1649-4.7240)	4.5555 (4.1319-4.9792)	4.8333 (4.5407-5.1260)	5 (5-5)	1.3333 (0.6337-2.0330)
SSM0210	*Haynaldia triticum* D.	Chris	3.6666 (3.0813-4.2520)	4.6667 (4.2242-5.1092)	4.7778 (4.4165-5.1391)	5 (5-5)	1.7222 (0.72265-2.7219)
SSM0237	*Ae. bicornis*	Chris	1.7222 (1.0433-2.4011)	3.3333 (1.6777-4.9889)	3.4999 (2.1588-4.8412)	4.6667 (3.8098-5.5235)	1.05556 (0.1869-1.9242)
SSM0241	*Ae. longissima*	Chris	2.5555 (1.9331-3.1781)	1.5 (0.5813-2.4187)	3.25 (2.3530-4.14698)	3.8472 (2.4173-5.277)	1.0833 (0.5674-1.5992)
SSM0242	*Ae. mutica*	Chris	3.4444 (2.7219-4.1670)	4.7222 (4.3783-5.0663)	4.6389 (4.2188-5.0589)	4.4999 (4.0307-4.9693)	1.1111 (0.3237-1.8985)

Table 3.5. Average disease scores of Chris, Selkirk, and 56-1 alloplasmic wheat lines to five *P. tritici-repentis* isolates/races; 331-9 (race 3), 86-124 (race 2), DW5 (race 5), ARcrossB10 (race not determined), and 88-1 (race 4) (continued).

Line	Cytoplasm Name	Nuclear Makeup	Mean Lower and Upper Confidence Intervals				
			331-9	**86-124**	**DW5**	**ARcrossB10**	**88-1**
SSM0254	*T. macha* 140191	Chris	4.0556 (3.6466-4.4645)	4.0833 (3.0710-5.0957)	4.5 (3.8871-5.1159)	4.8889 (4.6033-5.1745)	1.5556 (1.1319-1.9792)
Selkirk	*T. aestivum*	Selkirk	3.6111 (3.0086-4.2136)	4.5556 (4.0776-5.0335)	3.6667 (2.869-4.4644)	4.9445 (4.8016-5.08725)	2 (1.2023-2.7977)
SSM0003	*Ae. crassa* 6N	Selkirk	3.7222 (2.8822-4.5623)	4.5277 (3.8980-5.1576)	3.6111 (2.7354-4.4868)	4.8333 (4.5624-5.1043)	1.4722 (0.7866-2.1579)
SSM0004	*Ae. cylindrica* Cw	Selkirk	3.5278 (2.9177-4.1379)	4.16667 (3.4413-4.8921)	3.7222 (2.9194-4.5250)	4.75 (4.3398-5.1602)	2.1667 (1.5508-2.7826)
SSM0015	*T. dico* G1395	Selkirk	2.9445 (2.2304-3.6585)	4.4445 (4.0666-4.8223)	3.7917 (2.3331-5.2503)	4.8888 (4.6033-5.1745)	1.6667 (0.8098-2.5235)
SSM0016	*T. dico* G1453	Selkirk	3.8333 (3.0750-4.5917)	4.8889 (4.7083-5.0695)	3.9445 (2.9696-4.9193)	4.9445 (4.8016-5.0873)	2.2778 (1.4091-3.1465)
SSM0017	*T. dico* G1458	Selkirk	3.5556 (3.0289-4.0822)	4.30555 (3.638-4.9731)	4.2499 (3.6673-4.8327)	4.8333 (4.5407-5.1260)	2.1667 (1.5508-2.7826)
SSM0018	*T. dico* G1460	Selkirk	3.2778 (2.5302-4.02532)	5 (5-5)	3.6388 (2.5755-4.7023)	5 (5-5)	2.0556 (1.7116-2.3995)
SSM0020	*T. dico* G803	Selkirk	4.1666 (3.4413-4.8921)	4.9445 (4.8016-5.0873)	4.0556 (3.2155-4.8956)	5 (5-5)	2.0556 (1.9128-2.1984)
SSM0022	*T. dico* Okla 11140	Selkirk	3.5833 (2.6463-4.5204)	4.3889 (3.6748-5.1029)	3.8889 (2.8591-4.91871)	4.9167 (4.7703-5.063)	1.8333 (0.9836-2.6830)
SSM0039	*Ae. bicornis*	Selkirk	1.5277 (0.5358-2.5198)	3.5556 (2.2719-4.8393)	2.88888 (1.6637-4.1141)	4.1111 (2.8661-5.3561)	1.7778 (1.0552-2.50035)
SSM0045	*Ae. mutica*	Selkirk	3.5556 (2.9042-4.2069)	3.8333 (2.6891-4.9776)	3.6667 (2.8613-4.4720)	5 (5-5)	2.3333 (1.7480-2.9187)
SSM0054	*Ae. crassa*	Selkirk	3.5833 (2.3297-4.7837)	4.6111 (4.0507-5.1715)	4.4445 (3.5972-5.2917)	5 (5-5)	1.7778 (1.4922-2.0634)

Table 3.5. Average disease scores of Chris, Selkirk, and 56-1 alloplasmic wheat lines to five *P. tritici-repentis* isolates/races; 331-9 (race 3), 86-124 (race 2), DW5 (race 5), ARcrossB10 (race not determined), and 88-1 (race 4) (continued).

Line	**Cytoplasm Name**	**Nuclear Makeup**	**Mean Lower and Upper Confidence Intervals**				
			331-9	**86-124**	**DW5**	**ARcrossB10**	**88-1**
56-1	*T. durum* 56-1	56-1	4.6667 (4.1247-5.2086)	3.7222 (2.9747-4.46976)	4.0277 (3.4381-4.6175)	4.6667 (4.2242-5.1092)	2.97222 (2.0707-3.8737)
SSM0069	*Ae. longissima khaplis*	56-1	4.3333 (3.7076-4.9591)	3.5333 (1.9411-5.1256)	4.4445 (3.9665-4.9224)	4.5555 (4.1319-4.9792)	2.8055 (1.9457-3.6654)
SSM0076	*Ae. sharonensis*	56-1	4.5000 (3.9252-5.0748)	3.3333 (2.1216-4.5451)	4 (3.5053-4.4947)	3.8055 (3.0358-4.5753)	2.1944 (1.7744-2.6145)
SSM0085	*Ae. variabilis*	56-1	4.4445 (4.1588-4.7301)	3.5 (2.3137-4.6863)	3.6667 (3.3538-3.9796)	4.8611 (4.5041-5.2181)	2.7778 (1.8479-3.7077)
SSM0107	*T. dico* G1453	56-1	3.4445 (2.6570-4.2319)	2.63888 (1.4765-3.8013)	3.6666 (2.9003-4.4331)	3.8888 (2.9078-4.8700)	2.3889 (1.7566-3.0211)

Eight alloplasmic lines: SSM0192, SSM0193, SSM0194, SSM0195, SSM0202, SSM0208, SSM0210, and SSM0254, showed higher average disease scores compared to Chris, ranging from moderately susceptible, 3.49, to susceptible, 4. The other five lines: SSM0196, SSM0198, SSM0206, SSM0241, and SSM0242, had lower average disease ratings and were recorded ranging from moderately resistant, 1.8, to moderately susceptible, 3.4 (Table 3.5).

Figure 3.5. Reaction of tan spot differential lines, and other selected lines, to *P. tritici-repentis* isolate 331-9 (race 3).

Figure 3.6. Reaction of parental line Chris and alloplasmic line SSM0237 to *P. tritici-repentis* isolate 331-9 (race 3), which were significantly different.

When Chris alloplasmic lines were inoculated with race 2 isolate 86-124, they had an average disease score rating of 4.45 (Table 3.5). Only one line, SSM0241, was significantly different (Fig. 3.7). This line had a resistant reaction with a rating of 1.5, compared to the parent which was susceptible at 4.45 (Fig. 3.8). The scores for the other lines were higher or lower average disease scores, compared to the parent, but were not significantly different based on the confidence intervals. Seven alloplasmic lines showed a susceptible reaction of 4.5, to a highly susceptible reaction of 4.72: SSM0192, SSM0193, SSM0194, SSM0195, SSM0208, SSM0210, and SSM0242 (Table 3.5). The other six alloplasmic lines: SSM0196, SSM0198, SSM0202, SSM0206, SSM0237, and SSM0254, showed lower average disease scores compared to Chris, starting at 2.86, moderately susceptible, and reaching 4.19, susceptible (Table 3.5).

Figure 3.7. Reaction of tan spot differential lines, and selected euplasmic and alloplasmic lines, to *P. tritici-repentis* isolate 86-124 (race 2).

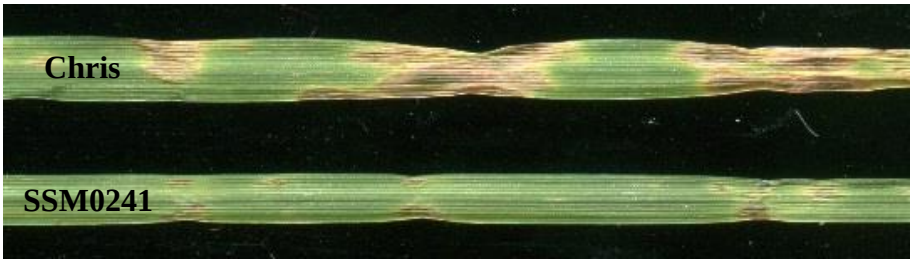

Figure 3.8. Reaction of euplasmic line Chris and alloplasmic line SSM0241 to *P. tritici-repentis* isolate 86-124 (race 2), which were significantly different.

Chris alloplasmic lines were also evaluated for their disease resistance against race 5 isolate DW5 (Table 3.5). With Chris, the mean disease score for DW5 was 4.57, highly susceptible (Fig. 3.9). Line SSM0241 was the only line that was significantly different from the parental line, with a moderately susceptible score of 3.25 (Fig. 3.10). Other Chris alloplasmic lines varied in their disease response from moderately susceptible to highly susceptible, but were not statistically different when compared to parental line Chris. Seven of the Chris alloplasmic lines: SSM0192, SSM0194, SSM0196, SSM0202, SSM0208, SSM0210, and SSM0242, expressed higher disease levels than Chris. Six other Chris alloplasmic lines: SSM0193, SSM0195, SSM0198, SSM0206, SSM0237, and SSM0254, recorded lower average disease scores, from 3.49 to 4.57 (Table 3.5).

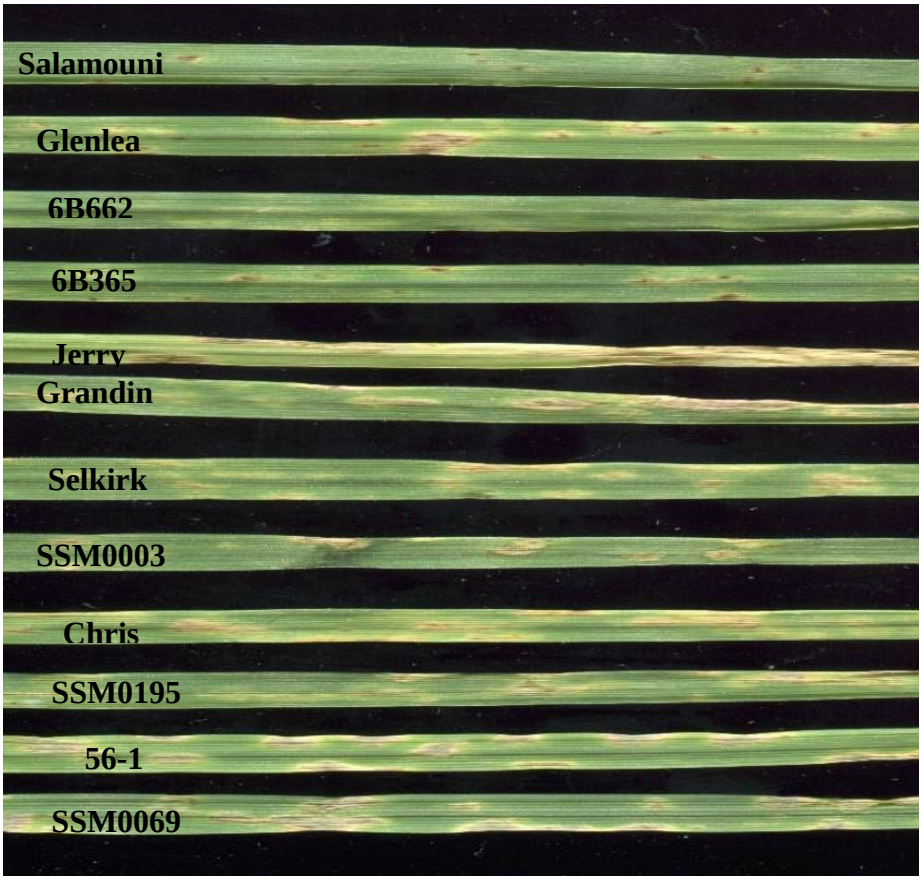

Figure 3.9. Reaction of tan spot differential lines, and selected euplasmic and alloplasmic lines, to *P. tritici-repentis* isolate DW5 (race 5).

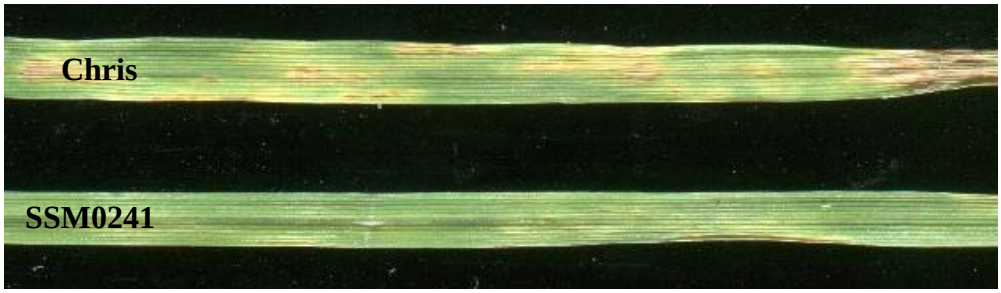

Figure 3.10. Reaction of euplasmic line Chris and alloplasmic line SSM0241 to *P. tritici-repentis* isolate DW5 (race 5), which were significantly different.

When Chris and its alloplasmic lines were evaluated for their sensitivity to ARcrossB10 (Table 3.5), Chris showed a highly susceptible reaction, with a mean disease score of 4.61 (Fig. 3.11). None of the Chris alloplasmic lines were significantly different when compared to the parent. Lines of this group appeared to vary in their resistance or susceptibility, and seemed to differ from the parental line, but were not significantly different. Eight Chris alloplasmic lines: SSM0192, SSM0193, SSM0194, SSM0206, SSM0208, SSM0210, SSM0237, and SSM0241, gave values higher than the parental mean disease score, ranging from 4.6 to 5 which is considered highly susceptible. Lines SSM0195, SSM0196, SSM0198, SSM0202, SSM0241, and SSM0242, expressed a susceptible reaction, ranging from 3.84 to 4.5 (Table 3.5).

The last isolate used for evaluation of Chris alloplasmic lines, 88-1, belongs to race 4 (Table 3.5). This race is considered avirulent since it does not produce any toxins. The parental line Chris expressed a highly resistant reaction, with an average disease rating score of 1.38 (Fig. 3.12). None of the Chris alloplasmic lines showed any significant differences when compared to the parental line. Some of the Chris alloplasmic lines: SSM0192, SSM0193, SSM0194, SSM0195, SSM0196, SSM0206, SSM0210, and SSM0254, expressed higher average disease scores, from 1.4 (highly resistant) to 2.5 (resistant). The other lines: SSM0198, SSM0202, SSM0208, SSM0237, SSM0241, and SSM0242, had lower average disease scores, which ranged from 1.08 to 1.38, and are all considered highly resistant (Table 3.5).

Figure 3.11. Reaction of tan spot differential lines, and selected euplasmic and alloplasmic lines, to *P. tritici-repentis* isolate ARcrossB10.

The Selkirk alloplasmic lines were evaluated with the same five isolates from the different races of *Ptr* (Table 3.5). For isolate 331-9, race 3, Selkirk's average disease score indicated moderate susceptibility to the isolate (Fig. 3.5). There was only one line, SSM0039, which showed significant differences, when compared to the parental line Selkirk. Line SSM0039 had an average disease score of 1.52, which is considered highly resistant (Fig. 3.13). Other lines showed either higher or lower disease ratings, but were not significantly different compared to Selkirk. Lines with higher average disease scores than Selkirk were: SSM0003, SSM0016, and SSM0020, with scores from 3.7 to 4.16, which are susceptible. Other lines

expressed lower average disease scores, from 2.94 to 3.58, but are still considered moderately susceptible, as was the parental line (Table 3.5).

Figure 3.12. Reaction of tan spot differential lines, and selected euplasmic and alloplasmic lines, to *P. tritici-repentis* isolate 88-1 (race 4).

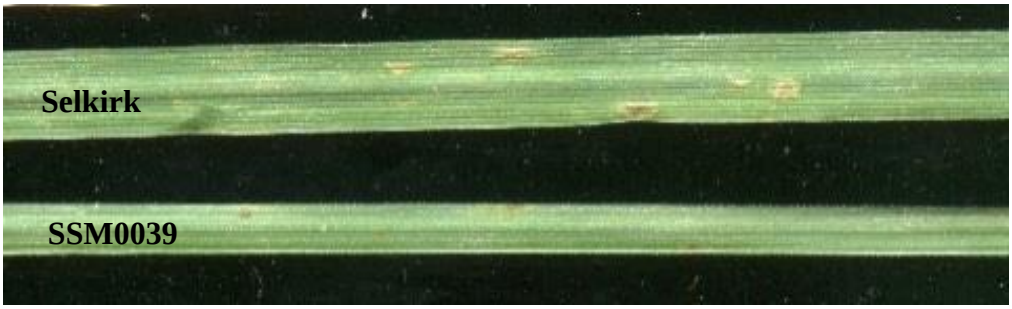

Figure 3.13. Reaction of euplasmic line Selkirk and alloplasmic line SSM0039 to *P. tritici-repentis* isolate 331-9 (race 3), which were significantly different.

The second isolate tested on Selkirk alloplasmic lines was 86-124, race 2 (Table 3.5). Average disease scores for Selkirk were susceptible at 4.57 (Fig. 3.7). Statistically, none of the lines showed significant differences when compared to the parent. However, the lines did vary in their reactions, and showed lower or higher disease intensity compared to the parental line. Lines showing higher mean disease scores and considered susceptible or highly susceptible were: SSM0016, SSM0018, SSM0020, and SSM0054. Lines with lower average disease scores which were moderately susceptible to susceptible were: SSM0003, SSM0004, SSM0015, SSM0017, SSM0022, SSM0039, and SSM0045 (Table 3.5).

With the race 5 isolate, DW5, the Selkirk alloplasmic lines were not significantly different from the parent (Table 3.5). Selkirk's average disease score was moderately susceptible at 3.67 (Fig. 3.9). Nine alloplasmic lines: SSM0004, SSM0015, SSM0016, SSM0017, SSM0018, SSM0020, SSM0022, SSM0045, and SSM0054, showed greater disease scores than the parental line, from moderately susceptible to susceptible, but were not significantly different. Two other lines, SSM0003 and SSM0039, had lower mean disease scores of moderately susceptible (Table 3.5).

Selkirk had an average disease score, when inoculated with ARcrossB10 (Table 3.5), of 4.94, which is considered highly susceptible (Fig. 3.11). None of the alloplasmic lines showed significant differences from the parental line when inoculated with this isolate. Eight lines:

SSM0015, SSM0016, SSM0017, SSM0018, SSM0020, SSM0022, SSM0045, and SSM0054, were highly susceptible to this isolate, based on the mean disease scores only. Three other lines: SSM0003, SSM0004, and SSM0039, had lower average disease scores, but were still considered susceptible to highly susceptible (Table 3.5).

The last isolate used to evaluate Selkirk alloplasmic lines was 88-1, race 4 (Table 3.5). The mean disease score for Selkirk was 2, which is resistant (Fig. 3.12). There were no significant differences among the alloplasmic lines when disease scores were compared to the parent, and all expressed a highly resistant to resistant reaction based on the average scores. Four alloplasmic lines: SSM0004, SSM0016, and SSM0017, and line SSM0045, were recorded with higher disease scores than the parental line. Several other lines also had average disease scores lower than their parental line: SSM0003, SSM0015, SSM0018, SSM0020, SSM0022, SSM0039, and SSM0054 (Table 3.5).

Alloplasmic lines of durum wheat line 56-1 made up the third group of lines to be evaluated with the tan spot isolates (Table 3.5). Starting with 331-9, race 3, the parental line disease reaction score was 4.67, which is considered highly susceptible (Fig. 3.5). None of the alloplasmic lines were significantly different from the parental line 56-1 in response to this isolate. All four lines SSM0069, SSM0076, SSM0085, and SSM0107, which belong to this parent gave a lower disease score than the parent, from moderately susceptible to a susceptible reaction (Table 3.5). With the second isolate, 86-124 (race 2), none of the alloplasmic lines of 56-1 were significantly different from the parent (Table 3.5). The parental line had an almost susceptible reaction, with an average disease score of 3.72 (Fig. 3.7). The other four lines all had lower disease reactions. Evaluation of disease level with isolate DW5 on parental line 56-1 resulted in a susceptible disease score of 4 (Fig. 3.9). One of the alloplasmic lines, SSM0107,

had a moderately resistant rating. The other three lines, SSM0069, SSM0085, and SSM0076, had equal or higher average disease ratings, which were susceptible. However, all these lines did not differ significantly from the parental line (Table 3.5). When isolate ARcrossB10 was used to inoculate this group, the alloplasmic and parental lines all showed susceptible reactions and did not have any significant differences between them (Table 3.5). The parental line's average disease score was 4.67 (Fig. 3.11). Three of its alloplasmic lines, SSM0069, SSM0076, and SSM0107, had lower disease scores. The last isolate used to evaluate this group for their resistance or susceptibility was 88-1. Line 56-1 showed a susceptible reaction with an average disease score of 2.97 (Fig. 3.12). The 56-1 alloplasmic lines expressed lower average disease scores, but were not significantly different from the parent (Table 3.5).

The last group of lines contained nuclear genes from both parental lines Chris and Selkirk (Table 3.6). Starting with isolate 331-9, Chris and Selkirk had moderately susceptible reactions, with average disease scores of 3.47 and 3.61, respectively. For this isolate, none of the lines were significantly different, when compared to both parents, however, line SSM0197 had an average disease score of 2.3, which is considered moderately resistant, but was not significantly different than either parent. The other two lines, SSM0191 and SSM0207, had higher disease ratings than both parents. The disease reaction with isolate 86-124, for both Chris and Selkirk, was susceptible. There were no significant differences when comparing the alloplasmic lines to both parents. Lines SSM0191 and SSM0197 had lower average disease scores compared to both parents, while the other line, SSM0207, had a greater mean disease score compared to both parents. Moderately susceptible to susceptible and reactions were expressed by Chris and Selkirk to isolate DW5, respectively. A significant difference was found only between line SSM0191 and Selkirk. Line SSM0191 had a higher mean disease score of 4.7, compared to Selkirk, with

3.67 (Fig. 3.14). Based on mean disease scores, the three lines had greater disease than both parents with DW5 (Table 3.6).

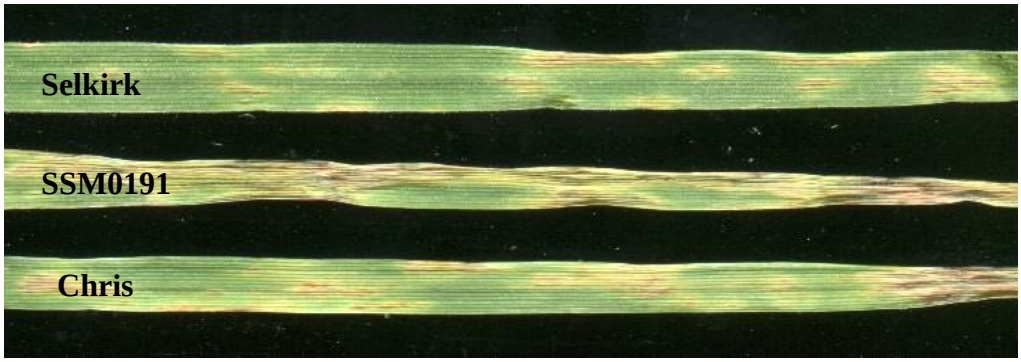

Figure 3.14. Reaction of two euplasmic lines, Chris and Selkirk, and alloplasmic line SSM0191 to *P. tritici-repentis* isolate DW5 (race 5), which were significantly different.

Chris and Selkirk were both susceptible and highly susceptible, respectively, to ARcrossB10 (Table 3.6). Again, the disease reaction of the alloplasmic lines compared to both parents was not significantly different. Lines SSM0191 and SSM0207 had lower mean disease scores compared to Chris, but not when compared to Selkirk. Line SSM0197 had an average disease score that was lower than both parents (Table 3.6). The last isolate used to evaluate these lines for disease reaction was 88-1. None of the three lines showed a significant difference when compared to both parents. Comparing the mean scores only, lines SSM0191 and SSM0197 had a lower mean score than Selkirk, but not lower than Chris, and with line SSM0207 the mean score was equal to Selkirk and higher than Chris (Table 3.6).

Table 3.6. Average disease scores and confidence intervals of alloplasmic wheat lines with a mixed nuclear background of Chris and Selkirk to five *P. tritici-repentis* isolates/races; 331-9 (race 3), 86-124 (race 2), DW5 (race 5), ARcrossB10 (race not determined), and 88-1 (race 4).

Line	**Cytoplasm Name**	**Nuclear Makeup**	**Mean Lower and Upper Confidence Intervals**				
			331-9	**86-124**	**DW5**	**ARcrossB10**	**88-1**
Chris	*T. aestivum*	Chris	3.47222 (2.49264-4.4517)	4.44444 (3.5145-5.3743)	4.555557 (4.19427-4.91684)	4.61111 (4.0506-5.1715)	1.3888 (0.8284-1.9493)
Selkirk	*T. aestivum*	Selkirk	3.61111 (3.00859-4.2136)	4.55555 (4.0776-5.0334)	3.666667 (2.86897-4.46436)	4.94444 (4.8016-5.0872)	2 (1.2023-2.7976)
SSM0191	*Ae. crassa*	CH+Sel	3.86111 (3.25102-4.4711)	3.83333 (3.1079-4.55871)	4.777778 (4.49215-5.06339)	4.80555 (4.573-5.0380)	1.94444 (1.8016-2.072)
SSM0197	*Ae. kotschyi*	CH+Sel	2.33333 (1.5589-3.10767)	3.388888 (2.0722-4.70553)	3.694443 (2.95720-4.43168)	4.16666 (3.3689-4.943)	1.38888 (0.8284-1.943)
SSM0207	*Ae. vavilovii*	CH+Sel	4.05555 (3.79222-4.3188)	4.66666 (4.1247-5.20859)	4.527777 (4.05305-5.0025)	4.8888 (4.708-5.069)	2.08333 (1.2573-2.9092)

Discussion

The disease response of 32 alloplasmic lines was measured with multiple isolates of the pathogens *P. nodorum* and *P. tritici-repentis*. The *P. nodorum* isolates, Sn2k and Sn4, and the five isolates of *P. tritici-repentis*, secrete different HSTs (Table 3.2). These HSTs are known to interact with the product of a specific sensitivity gene in the host, which leads to susceptibility to the necrotrophic pathogen. In the previous chapter, we tested the sensitivity of the alloplasmic lines to four of the toxins produced by these isolates in order to determine if the alloplasmic condition affects toxin sensitivity. We concluded that all alloplasmic lines responded to the toxins in a manner similar to the parental lines, and that toxin sensitivity or insensitivity is governed by genes located in the nucleus. In this chapter, we evaluated the disease response of the alloplasmic lines to spore inoculations of different isolates of *P. nodorum* and *P. tritici-repentis*, in order to investigate the effect of the alloplasmic condition on disease development compared to the euplasmic condition. Although many alloplasmic lines of Chris, Selkirk, and 56-1 expressed some disease variation to the isolates used, only a few showed a significant difference in disease susceptibility when compared to their respective euplasmic line (Tables 3.3, 3.4, 3.5, and 3.6).

P. nodorum isolate Sn2k is known to produce three toxins, while the isolate Sn4 produces five (Table 3.2). Two toxins are produced by both isolates, SnTox1 and SnToxA. Sn2k also produces SnTox5, and Sn4 produces SnTox2 and SnTox3. When Selkirk and Chris were tested for sensitivity to SnTox1, SnTox3, and Ptr/SnToxA, they were found to be resistant to SnTox1 and Ptr/SnToxA, but sensitive to SnTox3. The durum line 56-1 was sensitive to all three toxins. Although we were unable to obtain samples of SnTox2 and SnTox5 to test on these lines, Zhang et al. (2009) have shown that Selkirk is sensitive to SnTox2. Unfortunately, there are no reports

of testing done on these lines for sensitivity to the other toxins. Chris and Selkirk are resistant to SnTox1 and Ptr/SnToxA, leaving only SnTox5 to affect these lines when inoculated with Sn2k. Sn4 on the other hand, produces SnTox 2 and SnTox3 that could affect these lines. These differences in toxin production may partially explain the lower levels of necrosis seen when they were inoculated with Sn2k versus Sn4 (Figs. 3.1 and 3.2). The reduced sensitivity of Chris and Selkirk to Sn2k suggests that these varieties may not possess the sensitivity gene *Snn5*. The increased disease rating observed on Chris and Selkirk, when inoculated with Sn4, may be caused by the presence of SnTox2 and SnTox3. Zhang et al. (2009) showed that Selkirk is sensitive to SnTox2 by using specific markers for its sensitivity gene *Snn2*. At present, there are no reports on the sensitivity of Chris and 56-1 to SnTox2. In our experiments, 56-1 was sensitive to three of the toxins tested: SnTox1, SnTox3, and Ptr/SnToxA. In spite of this sensitivity, 56-1 gave a lower disease rating when inoculated with Sn4, compared to Sn2k. A possible explanation for this is that Sn2k secretes SnTox5, and that created the high susceptibility of the 56-1 parent and its alloplasmic lines, whereas Sn4 does not. Also, even if SnTox2 was produced by Sn4, it would not increase the disease intensity on 56-1 lines, since the sensitivity gene is located on chromosome D, which is not present in durum wheat. Lastly, as 56-1 is sensitive to SnTox3, it would only have the sensitivity gene on chromosome 5B, which has been proposed to have a lower affinity for the toxin (Zhang et al., 2011). This may reduce its response to Sn2k compared to the other two varieties.

Five different isolates from *P. tritici-repentis* were used in this study: 331-9, 86-124, DW5, ARcrossB10, and 88-1. Isolates 86-124, 331-9, 88-1, and DW5 belong to races 2-5 respectively, each secreting a single toxin (Table 3.2). ARcrossB10 secretes a necrosis toxin, that has not been characterized, as well as Ptr ToxC. With the race 3 isolate 331-9, the parental lines,

along with their alloplasmic lines, did not show the typical expanded chlorosis symptoms produced by ToxC, suggesting that they may not possess the Ptr ToxC sensitivity gene *Tsc1*. The differential line 6B365 showed the typical symptoms produced by the chlorosis toxin, demonstrating that the isolate could produce the toxin. The differential lines Salamouni, Glenlea, and 6B662 showed a resistant reaction when inoculated with 331-9. The necrosis seen in the parental lines and their alloplasmic lines may be caused by other, as yet to be characterized, pathogenicity factors. Both susceptible controls, Grandin and Jerry, showed highly susceptible reactions to the isolate, showing typical chlorosis and also necrosis (Fig. 3.5).

The race 2 isolate 86-124 showed necrosis on the parental lines Chris and Selkirk, along with their alloplasmic lines, even though these lines were resistant to Ptr ToxA. The durum line 56-1, along with its four alloplasmic lines, showed less necrosis than Chris and Selkirk, as well as lower mean disease scores, despite their sensitivity to Ptr ToxA. The production of Ptr ToxA was confirmed by the presence of necrotic lesions on the differential line Glenlea. The other differential lines, Salamouni, 6B662, and 6B365, all showed a resistant reaction with small dark flecks representing penetration points. Both Grandin and Jerry showed larger merged necrotic spots (Fig. 3.7).

DW5 belongs to race 5, which produces only Ptr ToxB, a chlorosis toxin. Infiltrations of Ptr ToxB into the parental and alloplasmic lines resulted in only Chris and its alloplasmic lines showing sensitivity to this toxin. This was confirmed by the observation of chlorosis symptoms on Chris and its alloplasmic lines when inoculated with DW5, similar to the chlorosis seen on 6B662 (Fig. 3.9). Again the other differential lines, Salamouni, Glenlea, and 6B365 had only small dark penetrations points. Grandin gave chlorosis symptoms, similar to 6B662, along with

some necrosis, and both Grandin and Jerry were very susceptible (Fig. 3.9). Jerry appeared to have more chlorosis and large necrotic lesions.

The isolate ARcrossB10 has not been assigned to a race, because although it does not possess the gene for Ptr ToxA, it can still produce necrosis on Glenlea. This isolate also produces the chlorosis toxin Ptr ToxC, and that was seen in differential line 6B365. Salamouni and 6B662 showed resistant responses to this isolate (Fig. 3.11). Chris, Selkirk, and their alloplasmic lines all gave necrosis symptoms to varying degrees. Of the three, Selkirk appeared to be the most sensitive. As mentioned before, Ptr ToxC has not been tested on the parental lines, so no information is available as to whether they contain the Ptr ToxC sensitivity gene.

The race four isolate 88-1 is reported to not produce any HSTs, and disease responses obtained from all lines tested were resistant (Fig. 3.12). Still, small necrotic lesions can be seen on Glenlea, and on Selkirk and 56-1, and their alloplasmic lines.

Only a few of the alloplasmic lines of Chris and 56-1 were significantly different in their disease response from the parental lines when inoculating with Sn2k or Sn4. The Selkirk alloplasmic lines also did not show a significant difference compared to the parental line, with the exception of SSM0039, which was significantly different when inoculated with Sn4 and when inoculated with *P. tritici-repentis* isolate 331-9. The cytoplasmic donor for SSM0039 was *Aegilops bicornis*. This line gave a susceptible response compared to the parental line's highly susceptible response with Sn4 (Fig. 3.3). When inoculated with 331-9, Selkirk was moderately susceptible, with an average disease score of 3.47, but SSM0039 was moderately resistant, with an average disease score of 1.52 (Fig. 3.6). If we consider only the disease ratings, the alloplasmic line SSM0237, the Chris alloplasmic line with *Ae. bicornis* cytoplasm, was moderately susceptible, but was not significantly different from the parental line with Sn4. Lines

SSM0039 and SSM0237 both had smaller second and third leaves compared to the other alloplasmic lines, which may have affected disease levels. The observed symptoms on both lines were mostly small dark spots with some spots being surrounded by a little chlorosis. It appears that the *Aegilops bicornis* cytoplasm's interaction with the nuclear genes have increased the resistance of Selkirk to Sn4 and 331-9.

Two of the alloplasmic lines derived from Chris, SSM0237 and SSM0241, showed a significant difference from the parental line. When inoculated with the isolate 331-9, race 3, SSM0237 showed a significant increase in resistance compared to the parent. Chris was rated as moderately susceptible, with an average disease score of 3.47, and SSM0237 was rated as moderately resistant, with a disease score of 1.72. The symptoms given by this line was a small penetration point or small necrosis (Fig. 3.6). Line SSM0237 contains the *Ae. bicornis* cytoplasm. When SSM0241, containing the *Ae. longissima* cytoplasm, was inoculated with isolates 86-124 and DW5, it showed a significant increase in resistance compared to Chris. The line showed small necrosis and penetration points surrounded with little chlorosis with 86-124 and DW-5, respectively (Figs. 3.8 and 3.10). With the other isolates there was not a significant increase in the resistance in any of the Chris alloplasmic lines. The 56-1 alloplasmic lines gave disease responses similar to the parental line among all isolates. The alloplasmic lines with mixed nuclei only showed significant differences with DW5. Line SSM0191, with cytoplasm donor *Ae. crassa*, was significantly different and had increased susceptibility compared only to Selkirk. The line showed chlorosis due to the effect of Ptr ToxB being produced by this isolate. Despite the impurity of the genetic background, this line still contains the sensitivity gene for Ptr ToxB and acted more like Chris than Selkirk. That also means the cytoplasmic genes may not be the reason behind the increase of the susceptibility (Fig. 3.14).

All 56-1 alloplasmic lines gave results similar to the parental line with all seven isolates used in this study. However, based only on the mean score, line SSM0076, with the *Ae. sharonensis* cytoplasm, was moderately susceptible compared to the parental line, when inoculated with isolate Sn2k.

The alloplasmic line SSM0197 has a mixed genetic background with nuclear genes from both Selkirk and Chris and the cytoplasm from *Aegilops kotschyi*. When inoculated with Sn2k, this line was significantly different only when compared to the Selkirk parent. The line was highly resistant and expressed only small dark penetration points, compared to Selkirk which showed a small amount of chlorosis and necrosis (Fig. 3.4). When Chris was inoculated with Sn2k it showed very little disease, similar to SSM0197. This line initially was a Selkirk line which was crossed with *Ae. kotschyi*, and then was backcrossed 13 times with Selkirk, followed by three times with Chris. Two other lines had increased susceptibility compared only with Chris. Lines SSM0191 and SSM0207, with *Ae. crassa* and *Ae. vavilovii* cytoplasms, respectively, showed resistant to moderately resistant reactions, compared with Chris which was highly resistant. Due to the mixed nuclear genetic background, it is difficult to assign the source of the resistance.

Some of the alloplasmic lines from Chris and Selkirk had the same cytoplasm but were derived from different accessions. When comparing the alloplasmic lines responses from the same parent, some lines with the same cytoplasm donor showed differences in their responses, and some were significantly different (Tables 3.3 and 3.5). Chris alloplasmic lines SSM0192 and SSM0193 had the same cytoplasm from different *Aegilops crassa* accessions. When inoculated with Sn2k, they gave different mean disease scores of 0.69 and 1.58, for SSM0192 and SSM0193, respectively, and were significantly different from each other. This indicates that even

though they contained the same cytoplasm, they had different nuclear-cytoplasmic interactions that led to different resistance responses. When inoculated with isolate Sn2k. lines SSM0194 and SSM0195 responded differently but were not significantly different from their parent. Both of these lines have *Ae. cylindrica* cytoplasm. Five alloplasmic lines of Selkirk had the same cytoplasmic donor but are from different accessions. Alloplasmic lines SMM0015, SSM0016, SSM0017, SSM0018, SSM0020, and SSM0022 all had *T. dicoccoides* as their cytoplasmic donor. All had similar average disease scores when they were inoculated with both *P. nodorum* isolates, and none one of them were significantly different from each other. When inoculated with the *P. tritici-repentis* isolates, all lines with the same cytoplasm showed no significant differences, but they varied in mean disease ratings.

Other studies have shown the effect of the substitution cytoplasm in wheat. In 2012, Burciaga evaluated the disease responses of some of the alloplasmic lines used in this study, in order to study the disease response when infected with different tan spot isolates and a mixture of four different races of the leaf rust pathogen *Puccinia triticina*. He found that an alloplasmic line from Chris, SSM0237 with *Ae. bicornis* as the cytoplasmic donor, had increased resistance compared to Chris, when inoculated with tan spot isolate Br15 and with the leaf rust mixture. Keane and Jones (1990) found that alloplasmic lines with *Ae. variabilis*, *Ae. squarrosa*, *Ae. ventricosa*, and *Ae. cylindrica* cytoplasms had significantly increased yield tolerance upon infection with *P. nodorum*. Although some of these cytoplasmic donors were used in creating the lines in this study, they did not show any significant changes in their disease resistance or susceptibility when compared to their parental line.

In summary, several alloplasmic lines in this study showed an increased resistance, compared to their parents, to different races of a pathogen and to different pathogens. Disease evaluations of two isolates of *P. nodorum*, and five isolates of *P. tritici-repentis*, showed cytoplasmic donors from *Ae. bicornis, Ae. kotschyi,* and *Ae. longissima* showed increased resistance compared to parental lines Chris and Selkirk. Only one line with the *Ae. crassa* cytoplasm exhibited an increased susceptibility. Of these three cytoplasms, *Ae. bicornis* and *Ae. longissima* increased disease resistance to multiple isolates from one pathogen, or gave resistance to isolates from both pathogens. This suggests that the cytoplasmic interactions between these cytoplasms and the wheat nucleus may be an additional source of disease resistance. The nuclear cytoplasmic interaction in lines SSM0039, SSM0237, and SSM0241 expressed a positive change in disease resistance against some of the wheat foliar pathogens. These lines should be further studied and genetically characterized to better understand the mechanism behind their resistance. The other alloplasmic lines with a mixed genetic background from Chris and Selkirk, SSM0197 and SSM0191, should also be genetically characterized to help understand if their resistance or susceptibility was caused by the alien cytoplasm, or because of other factors derived from the nuclear genes.

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APPENDIX A: SAS OUTPUT OF THE GLM ANALYSIS AND ANOVA TABLE FOR *P. NODORUM* ISOLATES SN2K AND SN4

Table A1. Analysis of variance of Selkirk alloplasmic wheat lines combined across three runs of *P. nodorum* isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	29.1645029	14.5822514	15.28	<.0001
Rep(Run)	15	28.1286667	1.8752444	1.97	0.0167
Line	12	144.4261393	12.0355116	12.61	<.0001
Race	1	788.2357452	788.2357452	826.18	<.0001
Run*Line	24	27.1476784	1.1311533	1.19	0.2508
Line*Race	12	77.5368087	6.4614007	6.77	<.0001
Run*Race	2	40.2710118	20.1355059	21.10	<.0001
Run*Line*Race	24	41.9471986	1.7477999	1.83	0.0106

Table A2. Analysis of variance of Chris alloplasmic wheat lines combined across three runs of *P. nodorum* isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	6.213156	3.106578	5.15	0.0062
Rep(Run)	15	17.710216	1.180681	1.96	0.0169
Line	15	253.866837	16.924456	28.05	<.0001
Race	1	1138.290380	1138.290380	1886.71	<.0001
Run*Line	30	36.129758	1.204325	2.00	0.0016
Line*Race	15	103.493332	6.899555	11.44	<.0001
Run*Race	2	2.679719	1.339860	2.22	0.1097
Run*Line*Race	29	23.171034	0.799001	1.32	0.1236

Table A3. Analysis of variance of 56-1 alloplasmic (durum) wheat lines combined across three runs of *P. nodorum* isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	0.1866370	0.0933185	0.08	0.9216
Rep(Run)	15	17.3180080	1.1545339	1.01	0.4466
Line	7	137.6527792	19.6646827	17.22	<.0001
Race	1	259.0078728	259.0078728	226.82	<.0001
Run*Line	14	10.9723243	0.7837375	0.69	0.7853
Line*Race	3	12.7606855	4.2535618	3.72	0.0127
Run*Race	2	2.2117454	1.1058727	0.97	0.3819
Run*Line*Race	6	6.6493426	1.1082238	0.97	0.4471

Table A4. Analysis of variance of alloplasmic wheat lines with a mixed nuclear background of Chris and Selkirk combined across three runs of *P. nodorum* isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	12.9114977	6.4557488	11.14	<.0001
Rep(Run)	15	6.5299242	0.4353283	0.75	0.7292
Line	5	142.2702450	28.4540490	49.10	<.0001
Race	1	332.5555612	332.5555612	573.84	<.0001
Run*Line	10	7.9236753	0.7923675	1.37	0.2001
Line*Race	5	83.6652108	16.7330422	28.87	<.0001
Run*Race	2	11.6022585	5.8011292	10.01	<.0001
Run*Line*Race	10	13.4644358	1.3464436	2.32	0.0140

APPENDIX B: SAS OUTPUT OF THE GLM ANALYSIS AND ANOVA TABLE FOR *P. TRITICI-REPENTIS* RACES/ISOLATES; 86-124 (RACE 2), 331-9 (RACE 3), 88-1 (RACE 4), DW5 (RACE 5), AND ARCROSSB10 (RACE NOT DETERMINED)

Table B1. Analysis of variance of Selkirk alloplasmic wheat lines combined across three runs of *P. tritici-repentis* races/isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	30.3473465	15.1736733	49.54	<.0001
Rep(Run)	3	0.2226535	0.0742178	0.24	0.8667
Line	12	34.6379713	2.8864976	9.42	<.0001
Race	4	381.5724883	95.3931221	311.45	<.0001
Run*Line	24	15.5866671	0.6494445	2.12	0.0028
Line*Race	48	26.3255909	0.5484498	1.79	0.0032
Run*Race	8	21.5638266	2.6954783	8.80	<.0001
Run*Line*Race	94	23.3434351	0.2483344	0.81	0.8727

Table B2. Analysis of variance of Chris alloplasmic wheat lines combined across three runs of *P. tritici-repentis* races/isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	42.5980300	21.2990150	57.45	<.0001
Rep(Run)	3	0.3494010	0.1164670	0.31	0.8152
Line	15	99.2170215	6.6144681	17.84	<.0001
Race	4	603.7533666	150.9383417	407.10	<.0001
Run*Line	30	17.5345806	0.5844860	1.58	0.0339
Line*Race	60	49.7166538	0.8286109	2.23	<.0001
Run*Race	8	10.7514524	1.3439316	3.62	0.0005
Run*Line*Race	119	46.1633881	0.3879276	1.05	0.3812

Table B3. Analysis of variance of 56-1 alloplasmic (durum) wheat lines combined across three runs of *P. tritici-repentis* races/isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	0.90597964	0.45298982	1.25	0.2929
Rep(Run)	3	0.12627664	0.04209221	0.12	0.9507
Line	5	12.48495859	2.49699172	6.87	<.0001
Race	4	73.24417036	18.31104259	50.35	<.0001
Run*Line	10	6.39201479	0.63920148	1.76	0.0809
Line*Race	20	23.97146019	1.19857301	3.30	<.0001
Run*Race	8	22.24259985	2.78032498	7.64	<.0001
Run*Line*Race	40	26.61362301	0.66534058	1.83	0.0101

Table B4. Analysis of variance of alloplasmic wheat lines with a mixed nuclear background of Chris and Selkirk combined across three runs of *P. tritici-repentis* races/isolates.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Run	2	7.6234597	3.8117298	11.31	<.0001
Rep(Run)	3	0.7222227	0.2407409	0.71	0.5462
Line	5	19.6512594	3.9302519	11.66	<.0001
Race	4	188.1676691	47.0419173	139.52	<.0001
Run*Line	10	6.0209737	0.6020974	1.79	0.0750
Line*Race	20	12.9120381	0.6456019	1.91	0.0209
Run*Race	8	9.5555336	1.1944417	3.54	0.0014
Run*Line*Race	40	9.1148337	0.2278708	0.68	0.9151