The Sensitivity of Canadian Wheat Genotypes to the Host-Selective Toxins Produced by *Pyrenophora tritici-repentis*

by

Van Anh Thi Tran

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
in
Plant Science

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Tan spot is a foliar disease of wheat caused by the fungus *Pyrenophora tritici–repentis*. Disease development is associated with the production of three host-selective toxins (Ptr ToxA, Ptr ToxB, and Ptr ToxC) that serve as fungal pathogenicity factors. The aim of this study was to investigate the sensitivity to each of these toxins in a representative collection of 100 Canadian wheat cultivars known as the VarComp population. Heterologously expressed, His-tagged Ptr ToxA and Ptr ToxB, and spore germination fluids possessing Ptr ToxC activity, were produced and used to screen the cultivars for toxin sensitivity. Ninety two of the 100 wheat cultivars tested were sensitive to at least one toxin, with 68% found to be sensitive to Ptr ToxA, 63% sensitive to Ptr ToxC, and 24% sensitive to Ptr ToxB. Eight cultivars were insensitive to all three toxins and may represent an important resource for tan spot resistance breeding activities.

Preface

This thesis is an original work by Van Anh Thi Tran. No part of this thesis has been previously published. Ms. Tran conducted all of the experiments described in this document, and wrote the first drafts of all chapters. The chapters were examined by Dr. Reem Aboukhaddour, Research Associate, who provided suggestions for improvement prior to submission to Dr. Stephen Strelkov, Professor and Ms. Tran's supervisor. Dr. Strelkov provided editorial revisions and suggestions on each chapter, which were then incorporated by Ms. Tran.

The work was supported by a NSERC Discovery Grant to Dr. Strelkov. The research project, of which this thesis is a part, received biosafety approval from the University of Alberta Biosafety Office, Environmental Health and Safety, May 06, 2009.

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Stephen Strelkov for his valuable guidance, advice and support during my study and research. His expertise in plant pathology and especially in tan spot disease of wheat significantly helped me in the completion of my thesis.

I also wish to acknowledge my supervisory committee members Dr. Dean Spaner and Dr. Kelly Turkington for their valuable suggestions on my project. I also would like to especially acknowledge Dr. Spaner for kindly providing the VarCormp population that was used in my study. I thank Dr. Urmila Basu for serving as an examiner in my defense.

I acknowledge Dr. Reem Aboukhaddour for helping to review my thesis and answering my research questions.

I would like to thank Ms. Rosa-Ileana Strelkov for technical training, assistance with protocol development as well as for providing research advice.

I acknowledge Dr. Yong Min Kim who created the *E.coli* tranformant containing *ToxB* which was used to produce N terminus His-tagged Ptr ToxB in my study.

I would like to acknowledge all of my labmates for their help during my research in the plant pathology lab.

I am grateful for the financial support provided by NSERC through a Discovery Grant to my supervisor.

Finally, I would like to thank my husband Chonggang Zhang and my son Harry Ruixin Zhang-Tran for their emotional support during my study.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction and literature review

1.1. Tan spot of wheat

Tan spot is an important foliar disease of wheat caused by the ascomycete fungus *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) (Da Luz and Hosford, 1980). This disease occurs worldwide, including throughout the major wheat growing regions of Australia, Canada, Brazil, the United States, and Argentina (Hosford, 1982; Rees *et al*, 1988; Strelkov and Lamari, 2003).

1.1.1. The history of tan spot and its causal agent

The casual agent of tan spot was initially studied on quack grass (*Triticum repens* L.) in Germany, with the fungus first identified as *Helminthosporium tritici-repentis* (Diedicke, 1902, 1904). In the United States, this fungus was also first described on *T. repens* in the early 1920s (Drechsler, 1923). The emergence of tan spot in Japan was reported by Nisikado (1928) and in India by Mitra (1931, 1934). In Canada, tan spot of wheat was reported on the wheat cultivar Marquis in 1927 (Conner, 1939). The pathogen was given different names in different countries, which caused confusion. For example, in Canada, the fungus was called *H. tritici-repentis* (Conners, 1939), as per Diedicke (1902, 1904), while in the United States it was referred to as *Pyrenophora tritici-vulgaris* (Barrus, 1942; Johnson, 1942). In addition, Valder and Shaw (1953) identified the causal agent of "yellow spot" in Australia as *H. tritici-repentis*. Tan spot and yellow spot of wheat are today regarded as the same disease. Further confusion ensued when *Pyrenophora trichostoma* was identified as a pathogen causing a wheat leaf spot disease in North Dakota (Hosford, 1971). Following comparison of the fungal propagules, it was concluded that

P. trichostoma and *P. tritici-repentis* are the same pathogen (Hosford, 1982). The causal agent of tan spot is now exclusively referred to by the telomorph name *P. tritici-repentis* and the anamorph name *D. tritici-repentis*.

1.1.2 The impact of tan spot

Tan spot of wheat has become an important foliar disease of wheat in Canada in recent decades. This increased prevalence of the disease has often been attributed to the widespread adoption of conservation tillage techniques by farmers. The fungus *P. tritici-repentis* can survive saprophytically on crop debris. Minimum or zero tillage systems can lead to the accumulation of a considerable amount of infected wheat residue on the soil surface, which serves as a source of primary inoculum in the next growing season (Rees and Platz, 1979). Nonetheless, a study by Bailey *et al.* (1992) in western Canada showed that the severity of leaf spot diseases (including tan spot) did not increase in zero or minimum tillage compared with conventional tillage, suggesting that other factors may also be involved. It is likely that the increased incidence of tan spot also may reflect the cropping of wheat in continuous or very short rotations, allowing the accumulation of infected host tissues and pathogen inoculum. Damage to the leaves caused by *P. tritici-repentis* infection results in a reduction in their photosynthetic capacity; when infection of the upper leaves occurs, the impact on grain fill and yields can be substantial (Wegulo *et al.*, 2006). Yield losses resulting from the development of tan spot generally range from 3 to 15%, but can be as high as 49% under conditions favourable for fungal growth (Rees *et al.*, 1982). In addition to yield losses, however, grain quality can also be affected.

The fungus *P. tritici-repentis* can also infect wheat kernels causing red smudge, a reddish discoloration of the grain, and/or black point or dark smudge, a blackening of the germ end of affected seeds (Francl and Jordahl, 1992; Fernandez *et al.*, 1997). Infection of the kernels results

in seed discoloration and reductions in seed quality. In Canada, all registered durum wheat cultivars are susceptible to red smudge (Fernandez *et al.*, 1997). In the infected seed, the anthraquinones catenarin and emodin can accumulate (Bouras and Strelkov, 2008). While toxicological studies on catenarin are lacking, emodin has been confirmed as a diarrheagenic, mutagenic, genotoxic, and cytotoxic compound (Liberman *et al*., 1980). Interestingly, catenarin is a red pigment, and its accumulation may contribute to the red smudge symptom (Bouras and Strelkov, 2008). The growth and production of anthraquinones by *P. tritici-repentis* is influenced by water activity and temperature. Optimal growth of *P. tritici-repentis* requires a high water activity (a_w = 0.99), suggesting that infection and mycotoxin contamination of the wheat kernels by this fungus occurs in the field rather than in storage (Bouras *et al*., 2009).

1.1.3. Symptoms of tan spot

On the leaves of susceptible wheat cultivars, tan spot is characterized by the development of two distinct symptoms, tan necrosis and extensive chlorosis. These symptoms result from the differential production of host-selective toxins by isolates of the fungus. These toxins, Ptr ToxA (necrosis-inducing), Ptr ToxB and Ptr ToxC (chlorosis-inducing) (Strelkov and Lamari, 2003) are discussed in greater detail below. Symptoms of infection appear on leaves as small oval or diamond-shaped necrotic and/or chorotic spots with a dark centre. If infection is severe, the lesions will coalesce and may cover most of or the entire surface of the leaf. In resistant cultivars, infection is localized and the fungus may cause only small brown flecks or spots which are harmless to the leaf (Hosford 1982; De Wolf *et al.*, 1998).

1.1.4. Host range

The fungus *P. tritici-repentis* has a wide host range including common or bread wheat (*Triticum aestivum* L.), durum wheat (*Triticum turgidum* L. var. *durum*), rye (*Secale cereale* L.) and numerous grass species. While common and durum wheat are regarded as the main hosts of *P. tritici-repentis* because of their economic importance, the fungus also has been identified and isolated from many other cereals and grass species. These include: *Psathyrostachys juncea* (Fisch.) Nevski (Russian wild-rye), *Leymus racemosus* (Lam.) Tzvelev (mammoth wild-rye), *Elymus angustus* (Trin.) Pilg. (altai wild-rye), *Elymus anadensis* L. (Canada wild-rye), *Critesion jubatum* (L.) Nevski or *Hordeum jubatum* (L.) Nevski (wild barley), *Pascopyrum smithii* (Rydb.) (western wheatgrass), *Agropyron desertorum* (Schult.) (desert wheat grass), *A. cristatum* (L.) (crested wheatgrass), *Bromus inermis* Leyss. (smooth bromegrass), and *Danthinia intermedia* Vasey (timber oatgrass) (Hosford 1971; Krupinsky, 1982). *P. tritici-repentis* overwinters on various secondary grass hosts and these hosts may serve as a pathogen reservoir between cropping seasons. In conclusion, the tan spot fungus has a wide host range and the pathogenicity of the isolates of the pathogen may differ on the various hosts (De Wolf *et al.*, 1998).

1.1.5. Disease cycle

P. tritici-repentis overwinters on infected wheat residues as sexual fruiting bodies called pseudothecia (Conners, 1939; Hosford, 1971). Light and high humidity are required for the development of the pseudothecia; in spring and early summer, pseudothecia release ascospores (sexual spores) that cause primary infection (Friesen *et al.*, 2003a). The optimum temperature for ascospore production is from 15 $^{\circ}$C to 18 $^{\circ}$C (Summerell and Burgess, 1989). While ascospores are spread by wind, their fairly large size (measuring 40 to 60 µm long x 18 to 25 µm wide) (Zillinsky, 1983) means that they cannot travel for very long distances (a maximum of

several centimeters). After landing on the leaf surface, the ascospores germinate, produce appressoria to penetrate into the host epidermal cells, and then grow intercellularly in the leaf mesophyll. Infection results in the development of the foliar lesions. Conidiophores, each bearing a single conidium (asexual spore), are produced on the lesions. The conidia are primarily wind-dispersed, but also may be rain-splased, and can travel for longer distances than ascospores (kilometers to tens of kilometers), and act as the secondary inoculum (Hosford, 1972; Morrall and Howard, 1975; Wright and Sutton, 1990; Schilder and Bergstrom, 1992). In wet periods under optimal temperature (21 ˚C), large numbers of conidia are produced and cause new infections (Francl, 1998). When the weather gets colder, pseudothecia form on wheat or other hosts and the pathogen overwinters on the host stubble (Conners, 1939; Morrall and Howard, 1975; Hosford, 1982). The disease cycle repeats throughout the growing season. The fungus also can survive as dormant mycelium in infected wheat seeds. When the seeds are planted, the mycelium begins to grow from the pericarp and infects the coleoptile. The fungus may then colonize the first two leaves of the seedling (Schilder and Bergstrorm, 1995).

1.1.6. Management of tan spot

Tan spot of wheat often occurs as part of a complex of leaf spot diseases in the field, which can make its identification and management more difficult (De Wolf *et al.*, 1998; Bockus and Shroyer, 1998). To manage tan spot, cultural and chemical control methods are commonly employed. The deployment of tan spot resistant hosts is another strategy, but as will be discussed below, the availability of resistant cultivars is limited.

Historically, one of the main methods by which farmers had kept tan spot and other stubble-borne diseases under control had been through tillage of the soil. However, in order to protect the soil from erosion, conservation tillage systems started to become widely adopted in

North America beginning in the 1970s. Conservation tillage also helps to maintain soil moisture and can result in increased crop yields. It also leads, however, to the accumulation of large amounts of infected wheat stubble on the soil surface (Bockus, 1998). These wheat residues serve as a primary source of inoculum in the next growing reason, and thus the incidence of tan spot has increased significantly. Crop rotation is another cultural method that can decrease the damage caused by tan spot and involves increasing the interval between the planting of susceptible wheat crops in the same field, providing time for the decomposition of infected residues and reducing the level of inoculum (De Wolf *et al.*, 1998). It was suggested by Rees and Platz (1979) that crop rotation with a three-year break between wheat crops would decrease tan spot incidence. In another study, a two-year rotation consisting of wheat and barley helped to reduce tan spot severity relative to continuous wheat (Sutton and Vyn, 1990). In general, non-host crops, such as soybean and alfalfa (Hosford, 1971) or peas and flax (Bailey *et al.,* 1992), represent good choices for inclusion between successive wheat crops. Chemical control, through the application of foliar fungicides, is another effective approach for tan spot control. A study by Jørgensen and Olsen (2007) showed that pyraclostrobin, picoxystrobin, propiconazole and prothioconazole were the most effective fungicides for the management of tan spot in Denmark. However, it is important to optimize the rate and timing of fungicide application to ensure its greatest efficacy. Fungicides should be applied prior to or immediately after a rain or irrigation event because these conditions may allow infection of the flag leaf (Colson *et al.*, 2003). In wheat, the purpose of fungicide application is to protect the upper leaves, especially the flag leaf, from damage by the fungus. Therefore, the optimum fungicide application interval is between the booting and fully headed developmental stages (Bockus, 1998). Making fungicide spray decisions, i.e., to spray or not to spray a particular crop, can be difficult. No established tan spot

forecasting systems are available. However, the application of a product might be prudent under wet and warm conditions favorable for the development of tan spot (Colson *et al.*, 2003). Indeed, the application of fungicides represents a very effective strategy for reducing the severity of leaf spot diseases and increasing yields under tight rotations in western Canada (Bailey *et al.,* 2000). In order to justify the application of a fungicide, the returns obtained from its application should be greater than the costs associated with its application. The cropping of resistant wheat cultivars is one of the most effective and low cost methods to avoid tan spot (De Wolf *et al.*, 1998; Gamba *et al.*, 1998). However, there are only a few registered cultivars with high levels of tan spot resistance (De Wolf *et al.*, 1998). In Australia, most wheat cultivars were found to be susceptible to tan spot (Rees *et al.*, 1988), with a few cultivars showing partial resistance (Rees and Platz, 1989; Wilson and Loughman, 1998). In Canada, only a limited number of wheat cultivars have resistance to tan spot (Lamari *et al.*, 2005). Efforts are underway to develop additional tan spot resistant wheat cultivars, and breeders are trying to identify resistance genes and quantitative trait loci (QTLs) for resistance (Faris *et al*., 2013). In addition, another strategy for developing tan spot resistant cultivars might be to eliminate from wheat, through traditional breeding techniques, the genes conferring sensitivity to the HSTs produced by *P. tritici-repentis*. Such an approach would be effective, however, only if no other uncharacterized pathogenicity or virulence factors are produced by fungal isolates.

1.1.7. The infection process

Typically, an ascospore or a conidium of *P. tritici-repentis* lands on a wheat leaf and germinates, forming a germ tube that then produces an appressorium and a penetration peg (Larez *et al*, 1986). Studies showed that 65% of conidia had germinated by 3 hours after inoculation, and about 95% had geminated after 6 hours (Larez *et al.*, 1986; Amaike *et al.*, 2008). The conidial

germination rates, number of germ tubes per conidium and production of appressoria increased under cool temperatures and high relative humidity (Hosford *et al.*, 1987). The penetration process involves enzymatic hydrolysis followed by mechanical puncture of the leaf surface (Dushnicky *et al.*, 1996). The penetration peg is formed below the appressorium, and can enter an epidermal cell directly or through a stoma, forming a vesicle (Dushnicky *et al.*, 1998a). The penetration process can be completed in as little as 3 hours after the spores land on the leaf surface (Dushnicky *et al.*, 1996). Following penetration, the fungus often produces one or two intracellular vesicles from the penetration peg (Larez *et al.*, 1986; Dushnicky *et al.*, 1996; Dushnicky *et al.*, 1998a). In some cases, no vesicles are formed, but hyphae still can grow intracellularly within the epidermal cells (Larez *et al*, 1986). In the initial stages of infection, *P. tritici-repentis* attacks both susceptible and resistant hosts and causes intercellular damage (Larez *et al.*, 1986). However, the number of papillae (localized wall thickenings on the inner surface of the plant cell walls at fungal penetration sites) formed was found to be higher in resistant versus susceptible cultivars (Dushnicky *et al.*, 1996; Dushnicky *et al.*, 1998a).

At about 24 h after inoculation, secondary hyphae may be able to extend into the mesophyll layer (Dushnicky *et al.*, 1996; Dushnicky *et al.*, 1998a). Fungal growth in the mesophyll, unlike in the epidermis, is intercellular. In resistant cultivars, the mesophyll cells surrounding the infection site and intercellular hyphae appeared thickened, diminishing the space in between cells (Dushnicky *et al*, 1998a). Lignification of the cell walls in resistant cultivars may contribute to the inhibition of hyphal growth and the thickening of the leaf cells at the infection site and in surrounding areas. The level of damage observed in resistant and susceptible host genotypes begins to be detectably different by fluorescence microscopy at 96 hours following penetration (Dushnicky *et al*., 1998a). In resistant genotypes, fungal invasion of the

mesophyll is stopped, resulting in the development of just a few small flecks or lesions on the leaves (Dushnicky *et al.*, 1996; Dushnicky *et al.*, 1998b). In susceptible hosts, however, fungal colonization of the mesophyll continues until the hyphae reach the large vascular mid-veins of the host (Larez *et al.*, 1986; Dushnicky *et al.*, 1998a). Host cells within the affected tissue eventually collapse and appear as a dead area on the leaf surface (Larez *et al.*, 1986).

1.1.8. Race classification

Isolates of *P. tritici-repentis* were initially grouped into four pathotypes based on their ability to differentially cause necrosis or chlorosis on particular wheat genotypes. Pathotype 1, the most common pathotype, could induce both necrosis and chlorosis ($nec^{+}chl^{+}$), pathotype 2 induced necrosis only ($nec^{+}chl^{-}$), pathotype 3 induced only chlorosis ($nec^{-}chl^{+}$), whereas pathotype 4 caused neither symptom ($nec^{-}chl^{-}$), (Lamari and Benier, 1989a). This classification system was limited to a maximum of four groups (based on the presence or absence of two symptoms) and could not accommodate new virulence patterns in the pathogen. This limitation was highlighted when Lamari *et al.* (1995) identified some isolates of *P. tritici-repentis* from North Africa that caused only chlorosis, and hence were pathotype 3, but caused this symptom in different host genotypes than pathotype 3 isolates from Canada. In order to accommodate the new virulence patterns, Lamari *et al.* (1995) proposed a race-based classification system for isolates of *P. tritici-repentis*. In this system, isolates are classified into races based on their virulence on a defined set of wheat differential lines or cultivars (Table 1-1). This system is now used almost universally by tan spot researchers worldwide (Lamari and Strelkov, 2010).

In the race-based classification scheme, the original pathotypes 1 to 4 became races 1 to 4, respectively, and the isolates exhibiting the new virulence pattern were classified as race 5 (Lamari *et al.*, 1995). While six hosts are included in the tan spot differential set, the genotypes

'Salamouni' and 4B1149 have been found to be resistant to all isolates of *P. tritici-repentis* characterized to date; in contrast, 'Coulter' is susceptible to all isolates except those classified as race 4. Hence, only three differentials ('Glenlea', '6B365' and '6B662') are effective for distinguishing the currently known races of the fungus (Lamari *et al.*, 2003). Race 1 isolates cause necrosis on 'Glenlea' and chlorosis on line '6B365', but are avirulent on line '6B662'. Isolates of race 2 cause necrosis on 'Glenlea' but are avirulent on 6B365 and 6B662, while race 3 isolates induce chlorosis on '6B365' but are avirulent on lines 'Glenlea' and '6B662'. Race 4 isolates do not cause any significant symptoms on any host genotype and are therefore regarded as avirulent. Race 5 isolates, which include the original chlorosis-inducing isolates from North Africa reported by Lamari *et al.* (1995), cause chlorosis on 6B662 but are avirulent on 'Glenlea' and 6B365. In 2002, another novel race, termed race 6, was identified from North Africa, which combined the virulence patterns of races 3 and 5 (i.e., isolates caused chlorosis on lines 6B365 and 6B662 but were avirulent on 'Glenlea') (Strelkov *et al.*, 2002). Surveys for tan spot in the wheat centre of diversity, including the Fertile Crescent and the Caucasus region, revealed the existence of two additional races of *P. tritici-repentis*, termed races 7 and 8 (Lamari *et al*., 2003; Lamari and Strelkov, 2010). Race 7 isolates cause necrosis on 'Glenlea' and chlorosis on line 6B662, combining the virulence patterns of races 2 and 5 (Lamari *et al*, 2003). Isolates of race 8 are virulent on all three of the effective differentials, causing necrosis on 'Glenlea' and chlorosis on lines 6B365 and 6B662, thereby combining the virulence patterns of races 2, 3 and 5 (Lamari *et al.*, 2003). The races of *P. tritici-repentis* differentially produce the host-specific toxins Ptr ToxA, Ptr ToxB and Ptr ToxC, as will be discussed below. In Canada and throughout the Americas and Australia, races 1 and 2 of *P. tritici-repentis* (sensitive to Ptr ToxA) are predominant (Strelkov and Lamari, 2010; Gamba *et al.*, 2012). Indeed, in a recent study of a

collection of 45 *P. tritici-repentis* isolates from Alberta, Canada, 62% of the isolates were classified as race 1, 36% were classified as race 2, and a single isolate (2%) was classified as race 3 (Aboukhaddour *et al.*, 2013). In contrast, races 7 and 8 were found to be common in the Caucasus and Fertile Crescent, regions which represent (at least in part) the centre of diversity of wheat, and hence also a centre of diversity of its associated pathogens (Lamari and Strelkov, 2010).

1.2. The involvement of host-selective toxins in the tan spot pathosystem

Host-selective toxins (HSTs) are defined as pathogenicity factors that cause toxicity and induce disease only in certain sensitive hosts (Scheffer and Briggs, 1981). Generally, HSTs cause symptoms only on hosts that are also susceptible to the fungal isolates that produce these toxins. These compounds have little if any effect in insensitive or resistant host genotypes (Pringle and Scheffer, 1964; Scheffer and Livingston, 1984). Many of the first HSTs to be characterized were secondary metabolites. In the tan spot pathosystem, however, at least two proteinaceous HSTs (Ptr ToxA and Ptr ToxB) have been identified (Strelkov and Lamari, 2003). A third HST, Ptr ToxC, appears to be a low molecular mass, non-protein molecule (Effertz *et al.*, 2002). All races of *P. tritici-repentis*, with the exception of race 4, produce at least one HST. Proteinaceous HSTs also have been recently identified from other fungal pathogens, such as *Stagonospora nodurum*, which produces SnTox1, SnTox2, SnToxA and Sn Tox3 (Friesen *et al.,* 2008b). Host selective toxins are now often referred to as necrotrophic effectors, because they confer virulence in necrotrophic fungal pathogens (Friesen *et al.,* 2008b). The characteristics and effects of Ptr ToxA, Ptr ToxB and Ptr ToxC produced by *P. tritici-repentis* are discussed in detail in the following section.

1.2.1. The necrosis-inducing toxin: Ptr ToxA

Ptr ToxA was the first of the Ptr toxins to be characterized (Lamari and Bernier, 1989c; Ballance *et al.*, 1996; Tuori *et al.*, 1995; Ciuffetti *et al.*, 1997; Sarma *et al.*, 2005). It was shown to serve as a pathogenicity factor for *P. tritici-repentis* (*sensu* Yoder, 1980) and when transformed into a toxin-non-producing isolate it conferred virulence upon that isolate (Ciuffetti *et al.*, 1997). The mature Ptr ToxA is a 13.2 kDa protein encoded in the host genome by a single-copy gene, *ToxA*.

The open reading frame (ORF) of *ToxA* is 534 base pairs (bp) long and consists of N and C domains. Two introns are found within the ORF: the first intron (55 bp) occurs in a non-translated leader sequence and the second intron (50 bp) in the C-terminus domain of the ORF (Ciuffetti *et al.*, 1997). The ORF encodes a pre-pro-protein 178 amino acids (aa) long with a molecular mass of 19.7 kDa (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). The pre-region of the ToxA protein (aa 1-22) represents a signal peptide for secretion. The pro-region (aa 23 to 60; the N-domain) has a function in folding of the molecule for the proper formation of a disulfide bond in the mature toxin structure. The C-domain (aa 61 to 178) corresponds to the 13.2 kDa mature toxin protein (Ciuffetti *et al.*, 1997).

The *ToxA* gene is located as a single copy on a 3.0 Mb B-like supernumerary chromosome (Lichter *et al.*, 2002; Aboukhaddour *et al*., 2009). *ToxA* was cloned and heterologously expressed, as both the full length gene and as only the N and C domains, in *Escherichia coli* (*E. coli*) with the expression vectors pPtrNEC and pTrc99A (Ballance *et al.*, 1996; Tuori *et al.*, 2000). Infiltration of the heterologously expressed protein into wheat leaves resulted in the development of necrotic symptoms on sensitive wheat genotypes and no symptoms on insensitive wheat genotypes. *ToxA* was also transformed into a *ToxA*$^{-}$ isolate of *P.*

tritici-repentis (race 4), where it was successfully expressed and conferred on that isolate the ability to induce necrosis on Ptr ToxA-sensitive wheat (Ciuffetti *et al.,* 1997).

Strelkov and Lamari (2003) stated that Ptr ToxA is produced by four races (1, 2, 7 and 8) of *P. tritici-repentis*, either by itself (race 2) or in combination with Ptr ToxC (race 1), Ptr ToxB (race 7) or both (race 8). A homologue of *ToxA* was identified in *S. nodurum*, cause of *S. nodurum* blotch of wheat (Friesen *et al.,* 2006). This gene encodes a HST that is very similar to Ptr ToxA, but which has been termed Sn ToxA to denote its *S. nodurum* origin. Eleven haplotypes of *ToxA* (encoding a HST termed Sn ToxA) were found in *S. nodurum* (SnToxA), while only one haplotype of *ToxA* have been identified in *P. tritici-repentis* (Friesen *et al.,* 2006). This led to the suggestion that *P. tritici-repentis* acquired *ToxA* from *S. nodorum* by horizontal gene transfer as recently as the 20th century (Friesen *et al.*, 2006).

According to Sarma *et al.* (2005), the three dimensional structure of Ptr ToxA consists of a single domain with a β-barrel fold that shows a solvent-exposed loop containing an RGD (arginyl-glycyl-aspartic acid) motif. This motif and the other amino acids present in the solvent-exposed loop are critical for the activity of Ptr ToxA (Meinhardt *et al.,* 2002; Manning *et al.,* 2008). The host mesophyll cells are postulated to possess a high affinity receptor for binding the RGD motif (Manning and Ciuffetti, 2005; Manning *et al.,* 2008). The high affinity receptor is encoded by the sensitivity gene *Tsn1* in the host and contains R gene related protein kinase, nucleotide binding and leucine-rich-repeat domains (Pandelova *et al.,* 2012). Following this binding, Ptr ToxA is taken up into the host cells in an endosome-like structure. Inside the cell, Ptr ToxA dissociates from the receptor and localizes to the chloroplast, where it interacts with ToxABP1 (ToxA Binding Protein 1), which is a chloroplast protein that is required for the proper functioning of photosystem II (Manning *et al*., 2007). The photosynthetic systems are affected

and the levels of reactive oxygen species (ROS) increase, resulting in cell death and the eventual development of foliar necrosis. The necrosis becomes visible in as little as 14 hours following toxin infiltration (Pandelova *et al.,* 2009). The necrosis symptom is light dependent; therefore, no symptoms are found in the dark (Manning and Ciuffetti, 2005). The accumulation of ROS in plant cells is a prerequisite for the development of necrosis (Manning *et al.*, 2009).

1.2.2. The chlorosis-inducing toxin: Ptr ToxB

The second host-specific toxin to be characterized from *P. tritici-repentis* was Ptr ToxB (Strelkov *et al.*, 1999). Like Ptr ToxA, the mature Ptr ToxB is also a small protein, in this case 6.5 kDa of mass (Strelkov *et al.*, 1999; Kim and Strelkov, 2007; Andrie and Ciuffetti, 2011). It is a heat stable, hydrophilic protein with a minimum specific activity of 14 nM (Strelkov *et al.*, 1999). Ptr ToxB is encoded by the *ToxB* gene. Unlike *ToxA*, *ToxB* occurs as multiple copies in isolates of *P. tritici-repentis* that possess Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). The *ToxB* gene consists of a 261 bp ORF with no introns present (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). The gene encodes a 87 aa protein including a 23 aa signal peptide for secretion (Martinez *et al.,* 2004; Strelkov and Lamari, 2003). The signal peptide is cleaved, yielding a mature protein 63 aa in length. The number of copies of *ToxB* present in an isolate is correlated with the degree of pathogenicity of that isolate. Strongly virulent race 5 isolates of the fungus have been found to possess between 8-10 copies of *ToxB* (Martinez *et al.,* 2004; Strelkov *et al.,* 2006), while a very weakly virulent isolate has only two copies (Strelkov *et al.,* 2006). This indicates a dosage effect, which has been confirmed by *ToxB* gene silencing in a highly virulent isolate of *P. tritici-repentis* (Aboukhaddour *et al.,* 2012); the extent of gene silencing in the knock-down strains was accompanied by corresponding decreases in virulence. In addition to its occurrence in Ptr ToxB-producing isolates of the fungus, homologs of *ToxB* have also been

identified, generally as single-copies, in races 3 and 4 isolates possessing no Ptr ToxB activity (Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006).

The chlorosis caused by Ptr ToxB is strictly light-dependent. In addition, the toxin did not have an effect on the synthesis of chlorophyll, but instead appeared to cause chlorophyll degradation (Strelkov *et al.*, 1998). Photosynthesis, as measured by infra-red gas analysis, declined significantly following toxin treatment of Ptr ToxB-sensitive wheat leaves, suggesting that the toxin directly or indirectly inhibits this process (Kim *et al.*, 2010). Photosynthetic rates declined prior to the development of detectable chlorosis. A proteomic comparison of Ptr ToxB vs. buffer treated leaves showed that this decline is accompanied by changes in the abundance of proteins involved in the light reactions of photosynthesis, the Calvin cycle, and the stress/defense response (Kim *et al.*, 2010). Treatment with some ROS scavengers also prevented the development of chlorosis in Ptr ToxB-treated leaf segments (Strelkov *et al.*, 1998). Collectively, these results suggest that Ptr ToxB directly or indirectly inhibits photosynthesis in sensitive wheat leaves, leading to oxidative stress and, ultimately, the photooxidation of chlorophyll molecules (Strelkov *et al.*, 1998; Kim *et al.*, 2010). It is this loss of chlorophyll that results in the typical foliar chlorosis or yellowing associated with Ptr ToxB. Additional research will be needed to identify the specific target or receptor for Ptr ToxB in sensitive wheat.

1.2.3. Another chlorosis inducing host-specific toxin: Ptr ToxC

In addition to Ptr ToxA and Ptr ToxB, some isolates of *P. tritici-repentis* produce a third HST, Ptr ToxC (Strelkov and Lamari, 2003). Ptr ToxC also induces foliar chlorosis, but on different host genotypes than Ptr ToxB. Indeed, sensitivity to Ptr ToxB and Ptr ToxC in wheat has been shown to be independently inherited (Gama *et al.,* 1998; Gama and Lamari, 1998). Ptr ToxC has not been purified to homogeneity and remains the least well characterized of the HSTs produced

by *P. tritici-repentis*. Nonetheless, the little information that exists regarding its chemical nature suggests that it is not a protein, but rather it is a small, non-ionic, polar and heat stable compound (Effertz *et al.*, 2002). The gene(s) responsible for the biosynthesis of Ptr ToxC are unknown (Lamari and Strelkov, 2010), and its mode of action remains to be investigated.

1.2.4. The relationship between HST production and race classification of *P. tritici-repentis*

The necrosis and chlorosis symptoms that are selectively induced by the races of *P. tritici-repentis* on the three effective wheat differential hosts, 'Glenlea', 6B365 and 6B662, result from the differential production by those races of Ptr ToxA, Ptr ToxB and Ptr ToxC (Strelkov and Lamari, 2003; Lamari and Strelkov, 2010). 'Glenlea' carries a gene *Tsn1* conferring sensitivity to Ptr ToxA (Faris *et al.,* 1996; Lamari and Strelkov, 2010), so that any isolate producing this HST can infect and cause necrosis on this host. Line 6B365 carries a single dominat gene *Tsc1* conferring sensitivity to Ptr ToxC (Effertz *et al.,* 2002), and hence isolates that produce this HST can infect and cause chlorosis on this host. Finally, line 6B662 carries a gene *Tsc2* (Friesen and Faris, 2004) that confers sensitivity to Ptr ToxB, so that Ptr ToxB-producing isolates can infect and cause chlorosis on 6B662. Therefore, the races of *P. tritici-repentis* are defined by their ability to produce the three Ptr toxins, alone or in combination (Strelkov and Lamari, 2003). Race 1 isolates produce Ptr ToxA and Ptr ToxC, causing necrosis on 'Glenlea' and chlorosis on 6B365. Race 2 isolates produce Ptr ToxA, causing necrosis on 'Glenlea'. Race 3 isolates produce Ptr ToxC, causing chlorosis on line 6B365. Race 4 isolates produce no known active toxins, and do not cause significant symptoms on any of the differentials (i.e, they are avirulent or non-pathogenic). Race 5 isolates produce Ptr ToxB, causing chlorosis on line 6B662. Race 6 isolates produce both Ptr ToxB and Ptr ToxC, causing chlorosis on lines 6B662 and 6B365, respectively. Race 7 isolates produce Ptr ToxA and Ptr ToxB, causing necrosis on 'Glenlea' and chlorosis on

line 6B662. Finally, race 8 isolates produce all three HSTs (Ptr ToxA, Ptr ToxB and Ptr ToxC), and can therefore induce symptoms on all three of the effective wheat differentials. Since the compatible interaction (susceptibility) is mediated by recognition between a putative host target or receptor and a pathogen-produced HST or necrotrophic effector, the tan spot system follows the toxin model or inverse gene-for-gene model of host-pathogen interactions (Strelkov and Lamari, 2003), which is described below.

1.3. Resistance to tan spot of wheat

1.3.1. The inverse gene-for-gene or toxin model of host-pathogen interactions

In necrotrophic fungi, HSTs function as effectors that confer on the fungus the ability to cause disease (Ciuffetti *et al.*, 1998). The compatible interaction results when the HST produced by a fungus is matched by a corresponding 'sensitivity' or 'susceptibility' gene in the host (Table 1-2). This gene could encode a receptor or some other molecular target for the HST. This is known as the toxin or inverse gene-for-gene model of host-pathogen interactions because it is essentially the mirror image of the classical gene-for-gene model (Flor, 1971), in which recognition of a pathogen effector by a host gene product confers susceptibility. Wheat varieties that are sensitive to the toxins are susceptible to the toxin-producing isolates of the fungus. The absence of either toxin products or the host toxin-sensitivity gene will result in the resistant response (no disease). Therefore, the toxins produced by *P. tritici-repentis* act as pathogenicity factors for this fungus (Strelkov and Lamari, 2003).

1.3.2. Genes controlling resistance/susceptibility to tan spot of wheat

Susceptibility to *P. tritici-repentis* is controlled by host genes (Lamari and Bernier, 1989c; Lamari and Bemier, 1991; Gamba and Lamari, 1998). Qualitative genes associated with the wheat response to the HSTs (pure toxins or culture filtrates) were referred to as *Tsc* (tan spot

chlorosis) and *Tsn* (tan spot necrosis). As noted above, the wheat gene *Tsnl* confers sensitivity to Ptr ToxA and, as a consequence, susceptibility to Ptr ToxA-producing isolates of *P. tritici-repentis*. This gene is located on the long arm of chromosome of 5B in both durum and common wheat (Faris *et al.*, 1996). However, despite the apparent importance of *Tsn1* in determining resistance/susceptibility to races of *P. tritici-repentis*, it may not account completely for the host reaction to necrosis. In fact, Friesen *et al.* (2003b) found that the Ptr ToxA-*Tsn1* interaction accounted for 24.4% of the disease reaction in recombinant inbred lines which were derived from a cross of 'Kulm' (sensitive to Ptr ToxA) x 'Erik' (insensitive to Ptr ToxA). Another study from Australia showed that *Tsn1* explained 39% of the variation in disease development in one population ('Krichauff/Brookton') and 60% in another population ('Cranbook/Halberd') (Cheong *et al.,* 2004), highlighting the importance of the *Tsn1* – Ptr ToxA interaction in the reaction to tan spot disease. Nonetheless, based on these results, the authors suggested that sensitivity to Ptr ToxA may not be the only critical factor for determining susceptibility to the disease, but may influence its severity. Other virulence or pathogenicity factors may also play a role in tan spot development, depending on the genetic background of the host. Faris and Friesen (2005) investigated two race non-specific resistance QLTs, *QTs.fcu-1B* and *QTs.fcu-3B*, which are located on the short arm of chromosome 1B and 3B, respectively, and are associated with resistance to all four races of *P. tritici-repentis* that produce Ptr ToxA. It has been suggested that *Tsnl* and Ptr ToxA do not directly interact with each other, but perhaps a complex of host proteins play a role in the identification of Ptr ToxA by *Tsn1* (Faris *et al.,* 2013). Indeed, as discussed above, Manning and Ciuffetti, (2005) and Manning *et al.* (2008) showed that ToxABP1 interacts with Ptr ToxA in the chloroplasts.

Sensitivity to Ptr ToxB is controlled by the *Tsc2* gene which is located on the short arm of chromosome 2B (Friesen and Faris, 2004). The *Tsc2*-Ptr ToxB interaction has been found to account for 69% of the variation in disease development in response to race 5, in combination with other minor effects on chromosomes 2A and 4B (Friesen and Faris, 2004). However, some studies on the inheritance of resistance to race 5 (insensitivity to Ptr ToxB) appeared to produce contradictory results. Lamari and Bernier (1991) and Singh *et al*. (2008) reported that a single dominant gene is responsible for resistance, whereas Singh *et al.* (2010) presented data suggesting the control of resistance by a recessive gene. Nonetheless, a recent study of an F_2 population derived from a cross between 'Salamouni' (resistance to race 5) and 'Katepwa' (susceptible to race 5) showed clearly that a single dominant gene confers sensitivity to Ptr ToxB (Abeysekara *et al.*, 2010). As has been previously reported (e.g., Amaike *et al*., 2008; Aboukhaddour *et al.*, 2012), quantitative variation in the virulence of *P. tritici-repentis* isolates may also occur and reflect the amount of toxin produced. Moreover, catenarin and other secondary metabolites may contribute to the development of non-specific symptoms, contributing to the aggressiveness of virulent isolates and masking specific interactions between wheat and *P. tritici-repentis* (Bouras and Strelkov, 2008). For these reasons, it may be preferable to use purified toxin instead of conidial inoculation or culture filtrates to study the mode of inheritance of Ptr ToxB sensitivity/resistance (Abeysekara *et al.*, 2010).

Sensitivity to Ptr ToxC (susceptibility to races 1 and 3) was shown to be under the control of a dominant gene in some crosses, but was found to be incompletely dominant in crosses with other cultivars (Lamari and Bernier, 1991). By contrast, later studies suggested that resistance to race 3-induced chlorosis is conferred by a single recessive gene (Gamba and Lamari, 1998; Gamba *et al.,* 1998). Another study by Singh and Hughes (2006) showed that resistance to race

1-induced chlorosis was controlled by a dominant gene. These conflicting results might reflect the fact that the expression of race 1 and race 3-induced chlorosis is influenced by environmental factors (Strelkov *et al.,* 2002). One QTL (QTsc.ndsu-1A), which was associated with resistance to chlorosis-inducing isolates of races 1 and 3 of *P. tritici-repentis*, was identified on the short arm of chromosome 1A (Faris *et al.,* 1997; Effertz *et al.,* 2001). Effertz *et al*. (2002) suggested that resistance to Ptr ToxC is controlled by a recessive gene *tsc1*, and that the incorporation of *tsc1* and the resistance QTL *QTsc.ndsu-1A* is likely responsible for high resistance to extensive chorosis. Unfortunately, since Ptr ToxC has not been purified, direct studies on the sensitivity of wheat to this HST have not been possible, and sensitivity is inferred by the development of chlorosis following conidial inoculation with races 1 or 3. It is possible that this has contributed to some of the apparently contradicting results.

1.4. Canadian wheat and its reaction to tan spot

In Canada, wheat is produced mostly in the prairie provinces of Alberta, Saskatchewan, and Manitoba. These provinces accounted for an average of 90% of the total wheat grown in Canada since 1908 and wheat production in western Canada was approximately 30 million tones in 2013 (Statistics Canada). Spring hexaploid wheat (Triticum aestivum L.), winter hexaploid wheat (T. aestivum) and durum wheat (Triticum turgidum L. ssp. durum (Desf.)Husn.) are the three main types of wheat grown in western Canada. Among these, spring hexaploid wheat is the most widely cropped, accounting for 9 million hectares of a total of approximately 12 million hectares of wheat. Western Canadian wheat includes eight classes: Canadian Western Red Spring (CWRS), Canadian Western Amber Durum (CWAD), Canadian Prairie Spring Red (CPSR), Canadian Prairie Spring White (CPSW), Canadian Western Extra Strong (CWES), Canadian Western Soft White Spring (CWSWS) (McCallum and DePauw, 2008), Canadian Western Red

Winter (CWRW), and Canadian Western General Purpose (CWGP) (http://www.grainscanada.gc.ca/wheat-ble/classes/classes-eng.htm). Canadian Western Red Spring has been the predominant spring bread wheat class. A few key cultivars have been planted widely over long periods of time. For example, 'Red Fife' (the first CWRS cultivar) was grown in Ontario and brought to western Canada in 1870 (Dickenson, 1976), where it was the predominant variety until 1909. 'Red Fife' was replaced by 'Marquis' because of agronomic drawbacks such as late maturity, susceptibility to lodging, a tendency to shatter, and susceptibility to stem rust (Newman, 1928). 'Marquis' was earlier maturing and higher yielding than its parents and less susceptible to shattering (Morrison, 1960). 'Marquis' was a popular cultivar both in Canada and the United States from 1900s to 1935, representing over 90% of the spring wheat grown in Western Canada (Fraser and Whiteside, 1956). 'Thatcher' replaced 'Marquis' in 1935 because of its stem rust resistance (DePauw and Hunt, 2001), and was the predominant wheat cultivar in Canada between 1939 and 1968. Many new wheat cultivars were derived from these important cultivars to obtain higher yields, desirable agronomic characters, and better disease resistance and grain quality. 'Neepawa', 'Wascana' and 'Wakooma' were released and extensively grown in the 1970s, 'AC Barrie' in the 1990s, and 'Superb' in the 2000s because of their high grain yield. Wheat cultivars with good disease and insect resistance have often been rapidly adopted, for example as was the case with 'Rescue' and 'Lillian' that were released in response to wheat stem sawfly outbreaks. The present dominant cultivars in western Canada include 'Harvest,' 'Lilian', 'Stettler', 'Unity', 'CDC Go', 'Glenn', 'CDC Utmost' and 'CDC Stanley' (http://www.grainscanada.gc.ca/wheat-ble/harvest-recolte/2013/variety-variete/2013-classes-en.html). Besides these popular cultivars, which all belong to the CWRS class, cultivars from other wheat classes also are grown in western Canada.

In Canada, a few tan spot-resistant wheat cultivars have been grown, including 'AC Crystal', 'AC Foremost', 'AC Taber', 'AC Karma', and 'AC Barrie', but most are susceptible to this disease (Lamari *et al.*, 2005). The sensitivity/insensitivity of 86 current and historical CWRS wheat cultivars to Ptr ToxA and Ptr ToxB was examined in one fairly recent study (Lamari *et al.*, 2005). It found that most cultivars from the 1800s until the mid-20th century were sensitive to Ptr ToxA. However, this sensitivity disappeared from Canadian wheat cultivars in the 1950s only to be (inadvertently) reintroduced in the 1960s (Lamari *et al.*, 2005). Sensitivity to Ptr ToxA first appeared in the cultivar 'Red Fife' and was then transferred through Canadian wheat lines by the extensive use of backcrossing to maintain the 'Marquis'–'Thatcher' bread making quality. This sensitivity has remained present in most wheat cultivars ever since. This is of particular concern because in North America, races 1 and 2 of *P. tritici-repentis*, which are known to produce Ptr ToxA, are predominant among the eight races of the fungus (Lamari and Strelkov, 2010). Therefore, most of the wheat cultivars in Canada are susceptible to races 1 and 2 and sensitive to Ptr ToxA. Although Ptr ToxB-producing isolates are rarely found in Canada, the sensitivity of wheat cultivars to Ptr ToxB first appeared in the cultivar 'Thatcher' in 1934 and was subsequently transferred to many genotypes through backcross breeding programs. Sensitivity to Ptr ToxB has persisted in western Canadian cultivars for over 70 years (Lamari *et al.*, 2005). This represents a potential cause for concern, particularly if Ptr ToxB-producing isolates of *P. tritici-repentis* were to become more widespread.

1.5. Tan spot resistance screening

Screening for tan spot resistance is very important, since it provides knowledge on the susceptibility and the resistance of wheat to this disease. In the field, screening is challenging because of difficulties in differentiating tan spot from other foliar diseases, producing sufficient

amounts of inoculum to apply to the test lines, and producing conditions amenable to tan spot development. The appearance of symptoms can also be slow in inoculated plots, so field screening can be time consuming. Screening under greenhouse conditions at the seedling stage is more convenient, but may have its own limitations. Greenhouse screening provides the most favorable conditions for disease development and therefore tan spot incidence and severity may be higher than in the field. In addition, seedlings are typically screened in the greenhouse, which may not always reflect the reaction of the adult plant. In tan spot of wheat and other pathosystems that follow the inverse gene-for-gene model, screening with the relevant HSTs or culture filtrates containing the toxic principle(s) represents a convenient method to gain information about the likely disease reaction of plant accessions. The HSTs can be produced in large quantities (such as through heterologous expression of proteinaceous toxins) and rapidly applied to the seedlings, without the need to culture or inoculate the corresponding fungal strains. Sensitivity to particular HSTs maybe provide a good indication of the susceptibility to the fungal strains producing those toxins; material that is sensitive to an HST can be screened out, while the resistance of material that is insensitive could be confirmed by inoculation with the fungus under field and/or greenhouse conditions.

1.6. Objectives of the current study

Given the economic importance of tan spot of wheat and the enhanced understanding of this pathosystem that has developed over the last few decades, an opportunity presents itself to evaluate the reaction of an extensive collection of 100 wheat cultivars (known as the 'VarComp Population'), which have been grown in Canada over the past century, for sensitivity to Ptr ToxA, Ptr ToxB and Ptr ToxC. Wheat cultivars in this population were developed and released by different organizations, including Agriculture and Agri-Food Canada, the SeCan Association,

the University of Saskatchewan, and the University of Manitoba. The 'VarComp population' includes a large number of CWRS cultivars, as well as selected cultivars from the CPS, CWES, CWAD, CWSWS, Canada Western General Purpose (CWGP), and Canada Western Hard White Spring (CWHWS) classes.

The specific objectives of this thesis are: (1) to heterologously express Ptr ToxA and Ptr ToxB in *Escherichia coli*, and to produce spore germination fluids with Ptr ToxC acitivity; and (2) to use these toxin preparations to evaluate the sensitivity of the 100 wheat cultivars of the VarComp population to each HST. The information obtained on the toxin sensitivity of these cultivars may serve as resource for wheat breeding programs focused on tan spot resistance, and help to avoid the unintentional release of material carrying toxin sensitivity traits. Ultimately, this information also may contribute to our understanding of the reaction of Canadian wheat to tan spot, and provide data to complement additional studies currently underway with the VarComp population.

Table 1-1. Reaction of six wheat differential hosts to eight races of *Pyrenophora tritici-repentis* (adapted from Lamari *et al.*, 2003).

Host genotypes	***P. tritici-repentis* races**							
	Race 1	**Race 2**	**Race 3**	**Race 4**	**Race 5**	**Race 6**	**Race 7**	**Race 8**
Glenlea	S(N)	S(N)	R	R	R	R	S(N)	S(N)
6B662	R	R	R	R	S(C)	S(C)	S(C)	S(C)
6B365	S(C)	R	S(C)	R	R	S(C)	R	S(C)
Salamouni	R	R	R	R	R	R	R	R
Coulter	S(N)	S(N)	S(N)	R	S(N)	S(N)	S(N)	S(N)
4B1149	R	R	R	R	R	R	R	R

S (N) = susceptible (necrotic symptom); S (C) = suceptible (chlorotic symptom); R= resistant.

Table 1-2. The interaction between toxin-producing fungal isolates and toxin-sensitive and insensitive host plants in the inverse gene-for-gene model of host-pathogen interactions (adapted from Anderson *et al.*, 1999).

Toxin production	**Sensitivity of host**	
	S	**s**
Tox^+	+	-
Tox^-	-	-

S = sensitive host; s = insensitive host; Tox^+ = presence of toxin; Tox^- = absence of toxin; + = disease development; - = no disease.

Chapter 2: The sensitivity of Canadian wheat genotypes to the host-selective toxins produced by *Pyrenophora tritici-repentis*

2.1. Introduction

Tan spot of wheat, also known as yellow spot, is caused by the ascomycete fungus *Pyrenophora trititci-repentis* (Died.) Drechs. (anamorph: *Dreschslera tritici-repentis* (Died.) Shoem.) and is a destructive foliar disease of common and durum wheat worldwide. Yield losses caused by tan spot can be as high as 50% under conditions favorable for the disease (De Wolf *et al.,* 1998; Hosford, 1982). These losses result from a reduction in the photosynthetic area of the infected leaves, through the development of tan colored necrotic lesions and (or) extensive chlorosis (Lamari and Bernier, 1989a). In recent decades, there has been an increased incidence of tan spot in western Canada, which has been attributed in part to low diversity crop rotations (Bailey *et al.,* 1992; Bailey *et al.,* 2001) and a shift towards conservation tillage practices that retain *P. tritici-repentis* infected crop residues on the soil surface (Rees and Platz, 1992).

The chlorosis and necrosis symptoms associated with tan spot result from the activity of host-selective toxins that are differentially produced by isolates of *P. tritici-repentis*. Three host-selective toxins have been identified to date: Ptr ToxA, Ptr ToxB and Ptr ToxC, all of which serve as pathogenicity determinants for the pathogen (*sensu* Yoder, 1980). Ptr ToxA is a necrosis inducing 13.2 kDa protein encoded by the single-copy gene, *ToxA* (Ballance *et al.,* 1989; Tuori *et al.,* 1995; Ciuffetti *et al.,* 1997). Ptr ToxB, another proteinaceous toxin, is 6.5 kDa in mass and encoded by the multi-copy *ToxB* gene. This toxin causes chlorosis in sensitive wheat genotypes (Strelkov *et al.,* 1999; Lamari *et al.,* 2003; Strelkov *et al.,* 2006). The third toxin, Ptr ToxC, also causes chlorosis in sensitive wheat lines or genotypes, but causes this symptom on different host genotypes than Ptr ToxB (Strelkov and Lamari, 2003). Unlike Ptr ToxA and Ptr

ToxB, Ptr ToxC has been only partially purified and appears to be a low molecular mass, polar, non-ionic compound (Effertz *et al.,* 2002). Eight races of *P. tritici-repentis* have been described and are defined by their ability to differentially produce the Ptr toxins, which in turn determines their virulence on a wheat differential set (Lamari and Strelkov *et al.*, 2010).

Sensitivity to the Ptr toxins and susceptibility to the respective toxin-producing isolates of *P. tritici-repentis* co-segregate (Lamari and Bernier, 1991; Gamba and Lamari, 1998; Gamba *et al.,* 1998), and is controlled by single dominant and independently inherited genes in the host, with one gene for each toxin (Faris *et al.,* 1996; Gamba *et al.,* 1998; Stock *et al.,* 1996; Faris *et al.,* 2013). The dominant gene *Tsn1* is located on chromosome 5B and controls the sensitivity of wheat to Ptr ToxA (Faris *et al.,* 1996). Wheat genotypes that lack *Tsn1* are insensitive to Ptr ToxA and resistant to the Ptr ToxA-producing races (Anderson *et al.,* 1999; Friesen *et al.,* 2003b). Another dominant gene, *Tsc2*, is located on the short arm of chromosome 2B and controls sensitivity to Ptr ToxB (Friesen and Faris, 2004). Sensitivity to Ptr ToxC is controlled by the dominant gene *Tscl*, which is located on the short arm of wheat chromosome 1A (Effertz *et al.,* 2002). The development of necrosis or chlorosis as a result of toxin action is largely a qualitative response, with a one-to-one relationship observed between the Ptr toxins and matching sensitivity genes in the host (Lamari *et al.,* 2003). As such, compatibility is the basis for specificity in the wheat/*P. tritici-repentis* interaction, with the tan spot pathosystem following the toxin or inverse gene-for-gene model of host-parasite interactions (Lamari *et al.,* 2003).

Given the highly specific nature of the interaction between *P. tritici-repentis* and wheat, the development of resistant cultivars represents a durable, cost effective and environmentally safe way to manage tan spot, particularly when compared with alternative approaches such as the application of fungicides. However, a study from Australia found no complete resistance to tan

spot in a collection of 1,400 bread wheat cultivars and lines tested by inoculation with a suspension of field-collected conidia of *P. tritici-repentis* (Rees and Platz, 1990). In a more recent study from the United States, Singh *et al.* (2006a) found that only 10 of 126 elite hard red spring, white and durum wheat genotypes possessed high levels of resistance to races 2, 3 and 5 of *P. tritici-repentis* and were insensitive to Ptr ToxA and Ptr ToxB. In Canada, in addition to short crop rotation practices and conservation tillage, the emergence of tan spot has been linked to the inadvertent introduction of the *Tsn1* gene into many wheat cultivars (Lamari *et al.,* 2005). Indeed, the majority of wheat cultivars in Canada are susceptible to tan spot disease (Lamari *et al.,* 2005). In an assessment of the tan spot susceptibility of a collection of Canadian Western Red Spring wheat genotypes, Lamari *et al.* (2005) reported that many present day cultivars are sensitive to Ptr ToxA and Ptr ToxB. The sensitivity of these genotypes to Ptr ToxC, however, was not assessed. In the current study, a collection of 100 wheat cultivars formerly or currently grown in Canada (known as the 'VarComp population') was tested for sensitivity to Ptr ToxA, Ptr ToxB and Ptr ToxC, in order to gain further insights into the prevalence of toxin sensitivity, and by extension the likely resistance/susceptibility to *P. tritici-repentis*, of Canadian wheat.

2.2. Materials and methods

2.2.1. Plant and fungal material

Plant material and growing conditions. The VarComp population includes wheat genotypes of Canadian Western Red Spring (CWRS, 62 genotypes), Canadian Prairie Spring (CPS, 9 genotypes), Canadian Western Amber Durum (CWAD, 14 genotypes), Canadian Western Extra Strong (CWES, 6 genotypes), Canadian Western General Purpose (CWGP, 3 genotypes), Canadian Western Soft White Spring (CWSWS, 4 genotypes) and Canadian Western Hard White Spring (CWHWS, 2 genotypes). The cultivars in the collection were developed by various

Canadian wheat breeding programs (DePauw *et al.,* 1995; DePauw *et al.,* 2001; McCallum and DePauw, 2008). Seeds of these cultivars were provided by Dr. Dean Spaner (University of Alberta) for the current study. The wheat genotypes 'Salamouni', 'Glenlea' and 6B662 and 6B365, of the standard tan spot differential set, were also included as references. 'Salamouni' is insensitive to the all known Ptr toxins, while 'Glenlea', 6B662, and 6B365 are sensitive to Ptr ToxA, Ptr ToxB or Ptr ToxC, respectively.

The wheat cultivars in the VarComp population were tested for their sensitivity or insensitivity to the three HSTs (Ptr ToxA, Ptr ToxB and Ptr ToxC) produced by *P. tritici-repentis*. Wheat seeds were sown in 10-cm-diameter plastic pots filled with Sunshine potting mix (W.R. Grace and Co., Fogelsville, PA) at a density of 5 seeds per pot. The seedlings were maintained for 2 weeks in a growth cabinet at 21°C (day) and 18°C (night) with a 16 hour photoperiod, and were watered and fertilized as required (Lamari and Bernier, 1989). The second leaf of 2 week-old (3 leaf stage) seedlings was treated with Ptr ToxA, Ptr ToxB or Ptr ToxC as described in succeeding paragraphs.

Fungal isolates. Conidia of race 3 isolate 331-2 of *P. tritici-repentis* (Lamari *et al.,* 1989b), were generated for the production of spore germination fluids for Ptr ToxC. Briefly, fungal cultures were grown in Petri dishes filled with V8-PDA medium (150mL V8 juice, 10 g potato dextrose agar, 3g calcium carbonate, 10 g agar to 1 L with water) in darkness at room temperature until the colonies reached 4 cm in diameter (Lamari and Bernier 1989a). The cultures were flooded with sterile distilled water and gently flattened with the bottom of a sterilized glass test tube. The water was decanted and the colonies were incubated overnight under light at room temperature, followed by 24 h incubation in darkness at 14°C to induce conidia formation. The sporulating colonies were again flooded with sterile distilled water, and

the conidia were gently dislodged with a sterile wire loop. The spore suspensions were collected and the conidial concentration was adjusted to approximately 3000 conidia mL^{-1} with sterile distilled water. Spore concentrations were measured with a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Blue Bell, PA).

Mycelium of race 2 isolate 86-124 also was produced for RNA extraction purposes (see below). Briefly, five plugs (1 cm in diameter) were cut from actively growing colonies of the fungus and transferred to a 250 ml Erlenmeyer flask containing 100 ml of Fries' (Dhingra and Sinclair, 1986). The flasks were incubated in darkness at room temperature with no agitation for 10 days. The mycelial mats were collected by vacuum filtration through Whatman No. 4 filter (Whatman International Ltd.., Maidstone, UK) and the mycelia were washed three times with distilled water. The mycelial mats were then flash-frozen on dry ice and stored at -80 ˚C for future use.

2.2.2. Heterologous expression and production of Ptr ToxA

Extraction of total RNA and cDNA synthesis. Total RNA of isolate 86-124 was extracted from 100 mg of 10 day-old flash-frozen mycelium using an RNeasy Plant Mini Kit (Qiagen, Maryland, USA) according to the manufacturer's instructions. The total RNA concentration was measured with a ND 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). The first cDNA strand was synthesized from 500 ng of total RNA with a Superscript TM III reverse transcriptase kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The second strand was produced by PCR amplification with the first strand cDNA as the template. The reaction mixture consisted of 0.2 µM of each sense and antisense primer, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 2 µl of first strand cDNA template, 1X PCR buffer (20 nM Tis HCl pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$ and 0.04 U Platinum®*Taq* polymerase

(Invitrogen, Canada Burlington, Ont) in a 50 µl volume. The amplification cycle included an initial heat denaturation step at 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. Double-stranded cDNA served as the template for PCR amplification of the *ToxA* insert.

Primer design. Primers to amplify the *ToxA* gene were designed based on the sequence data available in GeneBank for isolate 86-124 (accession no. AF004369), using Primer 3 Software (Rozen and Skaletsky, 2000). Forward primer F (5'- AAAAAGCAGGCTTCGAAAACCTGTATTTTGAGGGC**GAT CCC GGT TAC GAA AT** 3') and reverse primer R (5'- AGAAAGCTGGGTA*CTA***AATTTTCTAGCTGCATTCTCCAA-**3') were used to amplify 520 base pairs (bp) of an N and C domain sequence of *ToxA* from isolate 86-124. The gene-specific portions of the primer sequences are shown in bold and a TEV protease cleavage site is underlined, with the stop codon indicated in italics. To keep the *ToxA* insert in frame with the attP1 and attP2 sites (Landy, 1989) of the vector pDONR™ 221 (Invitrogen Canada), two adapter sequences (attB1 and attB2) were included at the end 5' of the forward and reverse primers. Two adapter-primers, consisting of attB1 (5'-G GGG ACA AGT TTG TAC AAA AAA GCA GGC T-3') and attB2 (5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3'), were used to generate the complete sequence of the entry clone 'attB1-*ToxA*-attB2'.

PCR amplification of the *ToxA* insert. The *ToxA* opening reading frame from isolate 86-124, including its N and C domain sequence, was amplified using GATEWAY® Technology with Clonase™ II (Invitrogen Canada) as per the manufacturer's instructions. Briefly, *ToxA* was ligated between the adapters attB1 and attB2 and the combined sequence 'attB1-*ToxA*-attB2' was ligated into the pDONR 221 vector at attP1 and attP2, respectively (Invitrogen). In order to

create the attB1-*ToxA*-attB2 sequence, a two-step PCR was carried out. In the first step, the *ToxA* gene was amplified with the *ToxA*-specific primers. The PCR was conducted in a 50 µl volume, containing 200 nM of each primer, 200 µM of each dNTP, 50 ng of DNA template, 1X PCR buffer (20 nM Tris HCl pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, and 2 U Platinum®*Taq* polymerase (Invitrogen). The amplification cycle consisted of an initial denaturation step at 95 °C for 2 min, followed by 10 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30s. The second step of this two-step PCR helped to install the full attB1 and attB2 sequences into the amplicon that was produced in the first step of the PCR. In this second step, 5 µl of DNA template from the first step of the PCR was included in a reaction mixture (50µl total volume) that also contained 800 nM of each adapter primer (attB1 and attB2), 200 µM of each dNTP, 1X PCR buffer, 1.5 mM $MgCl_2$ and 2 U Platinum®*Taq* polymerase (Invitrogen). The amplification cycle consisted of an initial 1 min denaturation step at 95 °C, followed by 5 cycles at 94 °C for 15 s, 45 °°C for 30 s and 68 °C for 30 s, and another 20 cycles at 94 °C for 15 s, 45 °C for 30 s and 68 °C for 30 s. The 'attB1-*ToxA*-attB2' amplicon was resolved by agarose gel (1.5 %) electrophoresis, excised, and purified with a gel extraction kit (Qiagen) following the manufacturer's instructions.

Ligation, transformation and selection of constructs. The purified amplicon 'attB1-*ToxA*-attB2' was inserted into the attP1 and attP2 sites of the pDONR211 vector to create the entry clone attL1-*toxA*-attL2, and this entry clone was transformed into One Shot ®OmniMAX™ 2-T1^R chemically competent *E. coli* cells **(**Invitrogen) as per the manufacturer's protocol. The transformants were selected against 50 µg/ml Kanamycin in Luria – Mertani (LB) medium. A positive control was transformed with a pEXP7-tet vector (Invitrogen) and selected against 20 µg/ml of tetracycline in LB medium. The negative control was transformed to One Shot

®OmniMAX™ 2-T1R chemically competent *E. coli* cells without the plasmid. The entry clone containing the *ToxA* insert was purified from a 5 ml aliquot of a culture of positive transformants with a plasmid purification kit (Qiagen) as per the manufacturer's instructions and sequenced at The Molecular Biology Service Unit, University of Alberta. Entry clones containing the right sequence were ligated into a pDEST™ 17 vector (Invitrogen) for expression of hexahistidine (6-His-tagged) fusion protein. In this step, the *ToxA* constructs were inserted between the attR1 and attR2 recombination sites of the expression vector through the site-specific recombination system of bacteriophage lambda (Landy, 1989) and transformed into Transforming Library Efficiency® DH5α™ Cells according to the manufacturer's protocol (Invitrogen). The transformants were selected against 100µg/ml ampicillin in LB medium. The positive controls were transformed into the pENTR™-gus vector (Invitrogen). No plasmid was included for transformation of the negative controls. The expression vector was purified with a plasmid purification kit (Qiagen) and transformed into BL21-AI™ One Shot® Cells (Invitrogen) for protein expression. To confirm that the expression clones contained the correct insert, colony PCR analysis with *ToxA* specific primers was conducted. Amplification conditions consisted of an initial step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s, and a final extension at 72 °C for 10 min. In addition, the purified expression plasmid was sequenced at the Molecular Biology Service Unit, University of Alberta, to confirm the correct sequence and reading frame. The *ToxA* constructs were stored in Transforming Library Efficiency® DH5αTM Cells (Invitrogen) in 80% sterilized glycerol (200µl bacterial culture: 800µl glycerol) at -80°C until needed. A diagrammatic representation of the general procedure involved for the cloning of the *ToxA* gene is shown in Fig. 2-1.

Ptr ToxA expression and purification. Transformed *E. coli* cells (50µl) were grown in 5 ml of LB medium containing 100 µg/ml ampicillin and incubated on a rotary shaker at 200 rpm at 37 °C for 2.5 h. From this initial culture, a volume of 250 µl was transferred into a 250 ml Erlemeyer flask containing 100 ml of LB medium (Sambrook *et al.,* 1989) and 100 µg/ml ampicillin and incubated at 37 °°C for 3 h, at 250 rpm, until the cells reached the mid-log phase (optical density of approximately 0.4 – 0.6). L-arabinose (Sigma-Aldrich, New Jersey, USA) was added to the culture to a concentration of 0.2% (w/v) in order to stimulate the expression of Ptr ToxA, and the culture was incubated overnight at 37°°C. The cells were collected by centrifugation at 4700×g for 15 min and the pellets were stored at -20 °C until use. The frozen cell pellets were thawed on ice for 30 min and re-suspended in 10 ml of lysis buffer (10 mM phosphate buffered saline – PBS pH7.4; 500 µg/ml lysozyme; 1 mM phenylmethylsunphonylflouride (PMSF); 5 µg/ml DNase I; 5 µg/ml RNase A; 1% (v/v) Triton X-100) and placed on ice for 30 min with gentle mixing. The suspension was subjected to sonication for 3 min to assist in disruption of the cell membranes. The cell lysis solution was centrifuged at 27,000×g for 15 min at 4 °C and the supernatant was discarded. The pellet was re-suspended in denaturing buffer (100 mM $NaH_2PO_4.H_2O$; 10 mM Tris-HCl; 8M urea, pH 8.0; 20nM β-mercaptoethanol) and incubated at room temperature with gentle mixing for approximately 1.5 h (until the cell pellet was completely re-suspended). The suspension was centrifuged at 27,000×g for 10 min and the supernatant was retained. The re-solubilized protein solution was incubated with nickel-nitrilotriacetic acid (Ni-NTA) agarose resin in 30% (v/v) ethanol (Qiagen) for 1.5 h in a ratio of 10 ml protein solution: 6 ml Ni-NTA slurry. The solution containing His-tagged Ptr ToxA and Ni-NTA agarose resin was loaded into a Poly-Prep chromatography column (Bio-Rad) and the column was washed with a total volume of 15 ml of

washing buffer (100 mM $NaH_2PO_4.H_2O$; 10 mM Tris-HCl; 8 M urea, pH 8.0; 30 mM imidazole) under gravity flow. The His-tagged Ptr ToxA was collected by adding 10 ml of elution buffer (100 mM $NaH_2PO_4.H_2O$; 10 mM Tris-HCl; 8M urea, pH 8.0; 300mM imidazole) to the column, with the eluate collected in 50 ml Falcon tubes (Franklin lakes, NJ, USA) under gravity flow.

The purified His-tagged Ptr ToxA was refolded *in vitro* to ensure its proper activity. The protein in elution buffer was placed in 10,000 molecular mass cut-off dialysis tubing (Spectra/Por®, Spectrum Laboratories Inc., Rancho Dominguez, CA) and dialyzed against1 L of 6M urea overnight (16 h) at 4°C with gentle stirring. A total of 2 L of 25 mM Tris-HCl (pH 7.5) buffer was gradually added to the 6 M urea in 250 mL or 500 mL aliquots every 6 or 12 h, respectively, until the total volume reached 3 L. The His-tagged Ptr ToxA then was dialyzed against 2 L of 12.5 mM Tris-HCl (pH 8.0), 0.125 mM EDTA (pH 8.0) for 24 h at 4°C. After dialysis was complete, the solution was centrifuged at 27,000×g for 10 min to remove any precipitated protein, and the supernatant was stored at -80°C. The His-tagged Ptr ToxA was lyophilized and re-suspended in 50mM Tris-HCl and 0.5 mM EDTA (pH 8.0) for later use. A flowchart summarizing the major steps involved in the heterologous expression and purification of Ptr ToxA is illustrated in Fig. 2-2.

2.2.3. Production of Ptr ToxB

Transformed BL21-AI ™ *E. coli* cells containing a pDEST™17 expression vector with *ToxB* from isolate Alg3-24 of *P. tritici-repentis* and producing N terminus His-tagged Ptr ToxB (Kim and Strelkov, 2007) were obtained from S.E. Strelkov (University of Alberta) and used to produce pure Ptr ToxB by heterologous expression and affinity chromatography, as described by Kim and Strelkov (2007) with the exception that the Ptr ToxB was dialyzed against 12.5mM

Tris-HCl and 0.125 mM EDTA (pH8.0) instead of 5mM sodium acetate (pH4.6) in the final dialysis step.

2.2.4. Production of Ptr ToxC spore germination fluid

Preliminary research (N. Bouras and S.E. Strelkov, unpublished data) indicated that the host-selective toxic principle corresponding to Ptr ToxC is found in spore germination fluids produced by *P. tritici-repentis* isolate 331-2. Therefore, spore germination fluids were produced by inoculating leaf segments of the Ptr ToxC-sensitive wheat line 6B365 with a spore suspension of isolate 331-2 (generated as described above). Briefly, leaf segments 2-3 cm long were cut from 2-week-old seedlings of 6B365 and placed in 9-cm-diameter Petri dishes containing a piece of wet filter paper (Whatman No. 1, Whatman International Ltd., Maidstone, UK). A few drops of the conidial suspension (approximately 3000 spores/mL) were placed on the surface of each leaf segment with a micropipette, and the Petri dishes were incubated under light at room temperature for 48 hours. Following incubation, the spore germination fluids were gently collected with a micropipette and passed through a 0.22 µm syringe filter (Fisher Scientific, Ireland) to remove conidial and mycelial debris, and stored at -20°C until use.

2.2.5. Estimation of protein concentration and polyacrylamide gel electrophoresis

The concentration of Ptr ToxA and Ptr ToxB was determined with a commercial kit (Bio-Rad, Mississauga, Ont.) based on the method of Lowry *et al.* (1951), with bovine serum albumin included as a standard. The proteins were visualized by PAGE under denaturing conditions with sodium dodecyl sulfate (SDS) in a mini-PROTEAN® 3 cell apparatus (Bio-Rad) using the buffer system of Schagger and Jagow (1987). Proteins were separated on a 5 % stacking gel and 15 % separating gel with a thickness of 0.75 mm. After electrophoresis, gels were stained in 0.05 %

(w/v) Coomassie brilliant blue dye R-250 or G-250 to visualize Ptr ToxA and Ptr ToxB, respectively. The gels were de-stained in 10 % acetic acid.

2.2.6. Bioactivity of Ptr toxin preparations

The activity of the heterologously expressed Ptr ToxA and Ptr ToxB and of the spore germination fluids containing the putative Ptr ToxC was assessed on the leaves of wheat genotypes sensitive and insensitive to each toxin. The second leaf of wheat seedlings at the 3 leaf-stage (14-days-old) was infiltrated with an aliquot of approximately 100 µl of the appropriate solution using a Hagborg (1970) device. The activity of each toxin preparation was tested in a dilution series: Ptr ToxA was tested at concentrations of 0.001, 0.01, 0.1, 0.3, 0.6, 1.4 and 3.5 µM; Ptr ToxB at concentrations of 0.5, 1, 5 and 10 µM; and Ptr ToxC at dilutions of 1:10, 1:20 and 1:50 spore germination fluid: water. Leaves of all of the wheat genotypes tested also were infiltrated with water, 50 mM Tris HCl and 0.5 mM EDTA (pH 8.0) as controls. Previous reports on the activity of His-tagged Ptr ToxA and His-tagged Ptr ToxB indicated that the tags did not affect toxin activity, and hence they were not removed in the current study (Tuori *et al.,* 2000; Kim and Strelkov, 2007). The symptoms caused by Ptr ToxA, Ptr ToxB and Ptr ToxC were assessed every 24 h until the necrosis or chlorosis was severe on the sensitive wheat genotypes at the highest concentration tested for each HST.

2.2.7. Testing of the toxin-sensitivity of the VarComp population

Based on the preliminary assessments of toxin activity, concentrations of 10 µM His-tagged Ptr ToxB and 1.4µM His-tagged Ptr ToxA were selected to infiltrate the wheat genotypes of the VarComp population as described above. Control treatments were infiltrated with 50 mM Tris HCl and 0.5 mM EDTA (pH 8.0). Spore germination fluids with Ptr ToxC activity were

infiltrated into wheat leaves at a dilution of 1:20 (spore germination fluid: water). The sensitivity testing of the wheat genotypes in the VarComp population was conducted separately for Ptr ToxA, Ptr ToxB and Ptr ToxC. In each run of the experiment, four seedlings of each host genotype were infiltrated with a particular toxin, with the entire experiment repeated twice for all genotypes and toxins. In a few cases, individual plants of the same genotype were found to differ in their reaction to a given toxin (i.e., some plants were sensitive, others insensitive). In such cases, an additional 30 seedlings of each genotype were infiltrated with the HST in question to assess how many were sensitive or insensitive. The reactions of the wheat genotypes to the HSTs were evaluated every day over a period of 3, 8, and 17 days post-infiltration for Ptr ToxA, Ptr ToxB and Ptr ToxC, respectively.

2.3. Results

2.3.1. Heterologous expression of Ptr ToxA in *E. coli*

His-tagged Ptr ToxA was produced abundantly in *E. coli* by heterologous expression, with approximately 300 mg of lyophilized affinity-purified His-fusion protein obtained from 1 liter of culture. The heterologously expressed Ptr ToxA was found in the insoluble fraction. Various concentrations of glucose were added to the LB medium, but this did not increase the solubility of the protein (data not shown). The insoluble protein fraction was solubilized in denaturing buffer and was refolded *in vitro* and affinity purified with a Ni-NTA agarose resin. The purified His-tagged Ptr ToxA with its N- and C-terminus domains and TEV protease cleavage site was expected to have a mass of 21.07 kDa, and a band of approximately that size could be resolved by sodium dodecyl sulfate (SDS)-PAGE (Fig. 2-3A). The levels at which the His-tagged Ptr

ToxA was expressed did not increase substantially after more than 3 hours of incubation (Fig. 2-4).

2.3.2. Heterologous expression of Ptr ToxB and production of spore germination fluids with Ptr ToxC activity

His-tagged Ptr ToxB was successfully expressed in *E. coli*, with the protein re-solubilized and purified from the insoluble fraction. The expected mass of the His-tagged Ptr ToxB (including the TEV protease cleavage site) was 11.1 kDa and a protein of approximately this size could be visualized by SDS-PAGE (Fig. 2-3B). With respect to the generation of spore germination fluids from the Ptr ToxC-producing isolate 331-2 of *P. tritici-repentis*, approximately 50 ml of fluid was generated on the leaves of the wheat line 6B365.

2.3.3. Activity of the Ptr toxins

The ability of the His-tagged Ptr ToxA to cause necrosis in a highly specific manner was confirmed by infiltrating the protein into leaves of the Ptr ToxA-sensitive wheat 'Glenlea' and Ptr ToxA-insensitive wheat 'Salamouni'. At concentrations of 3.5 µM and 1.4 µM, the heterologously expressed toxin caused visible necrosis symptoms on 'Glenlea' by 24h post-infiltration, which became severe to very severe by 48 h to 72 h. Necrosis also developed by 24 h and became severe by 72 h following infiltration with 0.3 µM and 0.6 µM concentrations of the toxin. By contrast, at 0.1 µM, Ptr ToxA-induced necrosis did not become clearly visible until 72 h post-infiltration. At concentrations of ≤0.1 µM, necrosis did not develop until 72 h after infiltration, with the necrosis localized to the center of the infiltration site at concentrations of 0.01 µM and 0.001 µM (Table 2-1; Fig. 2- 5A). As expected, no necrosis developed on the Ptr

ToxA-insensitive wheat 'Salamouni' at any protein concentration. Infiltration with water or buffer alone also did not produce any symptoms on 'Salamouni' or 'Glenlea'.

The selective chlorosis-inducing activity of the heterologously expressed, His-tagged Ptr ToxB was confirmed by infiltration into the leaves of the Ptr ToxB-sensitive wheat line 6B662 and of the insensitive 'Salamouni'. By 72 h after infiltration, chlorosis developed on 6B662 at a protein concentration of 5 μM and was strongest at 10 μM (Fig. 2- 5B). By contrast, no chlorosis was observed when leaves of 6B662 were treated with Ptr ToxB concentrations of 0.5 μM or 1 μM. No chlorosis symptoms developed on 'Salamouni' (Fig. 2- 5B) after infiltration with any concentration of toxin. Leaves of 6B662 or 'Salamouni' infiltrated with buffer or water alone also did not develop any chlorosis.

The spore germination fluids of isolate 331-2 induced chlorosis in a host-selective manner on line '6B365' (chl+) and 'Salamouni' (chl-). The chlorosis induced by the spore germination fluids was visible by 9 days and become severe by 2 weeks after infiltration with dilutions of 1:10 and 1:20 spore germination fluid: water (Fig. 2- 5C). At dilutions of 1:50 and 1:100, no chlorosis was observed. No chlorosis developed on the Ptr ToxC-insensitive wheat 'Salamouni' following infiltration with any dilution of spore germination fluids (Fig. 2- 5C); similarly, seedlings of '6B365' or 'Salmouni' did not develop any symptoms following treatment with water alone.

2.3.4. General reaction of the VarComp population

The two independent repetitions of the experiment in which the genotypes of the VarComp population were infiltrated with heterologously expressed Ptr ToxA, Ptr ToxB, or spore germination fluids with Ptr ToxC activity, produced very consistent results. The three HSTs induced typical and distinct necrosis or chlorosis symptoms on sensitive genotypes (Fig. 2- 6),

while having no or very little effect on insensitive hosts. Most tested genotypes (83 genotypes) were homogenous in their reaction to each of the three HSTs. Nine wheat genotypes, however, appeared to give heterogeneous reactions to infiltration with a particular toxin. In general, of the 100 wheat genotypes in the VarComp population, 68 were found to be sensitive to Ptr ToxA, 63 were sensitive to Ptr ToxC, and 24 were sensitive to Ptr ToxB. These included seven genotypes that were sensitive to Ptr ToxA and Ptr ToxB, 30 that were sensitive to Ptr ToxA and Ptr ToxC, and two that were sensitive to Ptr ToxB and Ptr ToxC. Twelve genotypes were found to be sensitive to all three toxins. These included '5500 HR', 'AC Domain', 'AC Intrepid', 'Bhishaj', 'CDC Go', 'Goodeve VB', 'Journey', 'Neepawa', 'Minnedosa', 'Park', 'Snowstar' and 'Waskada'(Table 2-2). By contrast, eight of the 100 host genotypes were insensitive to all three Ptr toxins. Most of the insensitive genotypes belonged to the CPS class, including 'AC Crystal', 'AC Foremost', 'AC Taber', 'HY682', and '5700PR'. The other insensitive cultivars were 'AC Avonlea', 'Brigade' and 'Eurostar' of the CWAD class (Table 2-2). All of the wheat genotypes in the CWRS class were sensitive to at least one toxin (Tables 2-2 and 2-3), while all host genotypes classified as CPS or CWES were insensitive to Ptr ToxB. Most of the tested genotypes in the CWAD class also were insensitive to Ptr ToxB, with the exception of 'AC Morse' and 'Transcend' which were sensitive. Most cultivars tested were sensitive to at least one of the Ptr toxins regardless of year of release, with sensitivity to Ptr ToxA and Ptr ToxC generally more common than sensitivity to Ptr ToxB over most decades (Fig. 2- 7).

2.3.5. Sensitivity to Ptr ToxA

As noted above, 68 out of 100 tested genotypes in the VarComp population were sensitive to Ptr ToxA. In most cases, all plants of the same genotype exhibited the same reaction to infiltration with the toxin. However, in the case of 6 genotypes, the reaction to Ptr ToxA was inconsistent

among some of the seedlings. In such cases, an additional 30 seedlings of each genotype were assessed again. In this additional testing, 1 (3.3%) seedling of '5701PR', 2 (6.6%) seedlings of 'AC Vista', 1 (3.3%) seedling of 'CDC Osler', 10 (33%) seedlings of 'Columbus', 4 (13%) seedlings of 'Thatcher', and 4 (13%) seedlings of 'Park' were found to be sensitive to the heterologously expressed Ptr ToxA. For all other host genotypes, the sensitivity to Ptr ToxA and the severity of necrosis it induced were highly consistent both among seedlings and between different runs of the experiment.

In the case of genotypes classified as CPS wheat, '5702 PR' was found to be homogeneously sensitive to Ptr ToxA, '5701 PR' and 'AC Vista' were heterogenously sensitive to the toxin, while the remaining cultivars in this class ('AC Crystal', 'AC Foremost', 'AC Taber', 'HY 682', 'SY 985', '57000 PR') were insensitive to the toxin (Table 2-2). Five of the CWES wheat cultivars were sensitive to Ptr ToxA, while a sixth 'CDN Bison' was insensitive (Tables 2-2 and 2-3). All four cultivars in the CWSWS class and the two cultivars in the CWHWS class were sensitive to Ptr ToxA (Tables 2-2 and 2-3). In the CWGP wheat class, 'NRG003' and 'NRG010' were insensitive to Ptr ToxA, while 'Minnedosa' was sensitive to the toxin (Table 2-2). A large percentage of genotypes in the CWRS class also were sensitive to Ptr ToxA, including the first popularly grown cultivars in Canada: 'Red Fife' and 'Marquis' (Table 2-2). 'Neepawa' (released 1969) was found to be sensitive to Ptr ToxA, and this sensitivity was likely transferred to 'Columbus' (1980), 'Katepwa' (1991) and many modern wheat cultivars, for example 'Lovitt' (2002), 'Somerset' (2004) and 'Alvena' (2006). Other CWRS wheat cultivars released in the 1990s and 2000s, including 'AC Domain' (1993), 'Harvest' (2002), 'Kane' (2006), 'Super' (2001), 'CDC Go' (2004), and 'CDC Abound' (2007), also were sensitive to Ptr ToxA. By contrast, several other cultivars released over the last two decades, namely 'AC

Eatonia' (1993), 'AC Abbey' (1998), 'Lilian' (2003), 'AC Elsa' (1996), 'Infinity' (2004), 'CDC Alsask' (2005) and others (Table 2-2), were insensitive to the toxin.

2.3.6. Sensitivity to Ptr ToxB

Only 24 of the 100 wheat genotypes in the VarComp population were sensitive to the heterologously expressed Ptr ToxB. The severity of the chlorosis that developed following infiltration with the toxin varied among the cultivars, with 'Eatonia', 'Katepwa' and 'Harvest' developing the most severe chlorosis. As was the case with Ptr ToxA, most genotypes yielded very consistent responses to Ptr ToxB, both within and between runs of the experiment. The only exceptions were '5500 HR' and 'Journey', some seedlings of which developed chlorosis while others did not in the two main repetitions of the experiment. Additional testing of seedlings of each genotype in a third assessment confirmed the heterogenous nature of their response; 5 (16.7%) and 15 (50%) of 30 seedlings of each of '5500 HR' and 'Journey', respectively, were sensitive to Ptr ToxB (Table 2-2). All cultivars classified as CPS and CWES class were insensitive to Ptr ToxB, as were most cultivars in the CWAD, CWGP and CWSWS classes (Table 2-2). Indeed, the majority of the Ptr ToxB-sensitive cultivars were classified as CWRS wheat, including 'Thatcher' (1935), 'Park' (1963) 'Neepawa' (1969), 'Katepwa' (1981), 'AC Domain' (1993), 'Harvest' (2002), 'CDC GO' (2004) and others (Table 2-2). Nonetheless, a subset of relatively modern cultivars in the CWRS class were insensitive to Ptr ToxB, including 'AC Barrie' (1994), 'AC Cadillac' (1994), 'AC Elsa' (1996), 'Infinity' (2004) and 'CDC Alsask' (2005) (Table 2-2).

2.3.7. Sensitivity to Ptr ToxC

Sixty three of the 100 tested wheat genotypes in the VarComp population were sensitive to Ptr ToxC (or, at least, to spore germination fluids with Ptr ToxC activity), with the intensity of the resulting chlorosis varying between cultivars. As was the case with sensitivity to Ptr ToxA and Ptr ToxB, the reaction of nearly all of the cultivars to Ptr ToxC was highly consistent within and between runs of the experiment. Only seedlings of three genotypes ('CDC Walrus', 'Roblin' and 'Waskada') showed variable responses to this toxin. Further testing of an additional 30 seedlings of each cultivar revealed that 14 (46.7%) seedlings of 'CDC Walrus', 5 (16.7%) seedlings of 'Roblin', and 10 (33.3%) seedlings of 'Waskada' were sensitive to Ptr ToxC. All cultivars classified as CWHWS or CWSWS were sensitive to Ptr ToxC (Table 2-2). All cultivars classified as CWES also were sensitive to this toxin with the exception of 'CDC Rama'. By contrast, most cultivars in the CPS and CWAD classes were insensitive to Ptr ToxC, with the exception of 'AC Vista', '5701 PR' and 'SY 985' (CPS); 'CDC Verona', 'Commander' and 'Napoleon' (CWAD), which were sensitive (Table 2-2). In the CWRS class, most genotypes were found to be sensitive to Ptr ToxC, including 'Neepawa' (1969), 'Columbus' (1980), 'AC Domain' (1993), 'Super' (2001), 'KANE' (2006), 'CDC Bound' (2007), and 37 others (Table 2-2). Nonetheless, the CWRS cultivars 'Harvest' (2002), 'CDC Bounty' (2000) and a few others were insensitive. A group of fairly recent cultivars from the CWRS class was sensitive to Ptr ToxC, including 'AC Barrie' (1994), 'AC Cadillac' (1994), 'AC Elsa' (1996), 'Infinity' (2004) and 'CDC Alsask' (2005) ' (Table 2-2).

2.4. Discussion

In the present study, the sensitivity of 100 Canadian wheat cultivars to Ptr ToxA, Ptr ToxB and the putative Ptr ToxC was evaluated in a bioassay. The results revealed that 92 of these 100

cultivars were sensitive to at least one HST, while only eight were insensitive to all three toxins. Most cultivars were sensitive to Ptr ToxA (68%) and Ptr ToxC (63%), while only 24% of cultivars were sensitive to Ptr ToxB.

The activity of the toxin preparations used to treat the cultivars was tested and the heterologously expressed Ptr ToxA and Ptr ToxB, as well as the spore germination fluids with Ptr ToxC activity, were confirmed to cause necrosis or chlorosis in a highly specific manner. His-tagged Ptr ToxA, consisting of the N and C terminus domains of the native Ptr ToxA, induced necrosis even without the presence of the signal peptide, indicating that the signal peptide is not necessary for full toxin activity. A similar result was reported by Tuori *et al.* (2000), who also found that the activity of His-tagged Ptr ToxA consisting of the N and C terminus domains was similar to that of purified mature Ptr ToxA. Preliminary assessments revealed that the optimal active concentrations of His-tagged Ptr ToxA and Ptr ToxB needed to cause clear symptoms in the bioassays were 1.4 µM and 10 µM, respectively, suggesting a higher specific activity for Ptr ToxA. This may reflect differences in the modes of action of the toxins.

Ptr ToxA damages wheat cells rapidly and causes cell death (necrosis). It has been shown to bind with a high affinity receptor in host mesophyll cells via an Arg–Gly–Asp (RGD) motif found in Ptr ToxA (Manning *et al.,* 2008). The toxin is taken up into the mesophyll cells and once inside it dissociates from the receptor and localizes to the chloroplasts. In the chloroplast, Ptr ToxA interacts with ToxA Binding Protein 1 (ToxABP1), which is required for the proper function of photosystem II (Manning *et al.,* 2007). This is postulated to cause a disruption in photosynthesis, resulting in the accumulation of ROS that cause rapid cell death and the development of necrosis (Manning *et al.,* 2009).

By contrast, the host response to Ptr ToxB is slower and results in chlorosis. Ptr ToxB activity does not appear to involve a high affinity receptor and the toxin is hypothesized to have an extracellular site of action (Ciuffetti *et al.,* 2010). Ptr ToxB was suggested to act in the apoplast because it has attributes in common with apoplastic effectors, including a small mass, high cysteine content and resistance to protease degradation (Ciuffetti *et al.,* 2010). Nonetheless, Ptr ToxB activity also has been shown to involve an inhibition of photosynthetic processes and the production of ROS, which cause a degradation of chlorophyll molecules and the eventual yellowing of the leaf tissue (Strelkov *et al.,* 1998; Kim *et al.*, 2010). In the present study, the strongest chlorosis symptoms were induced following infiltration of the leaves with a 10 μM concentration of His-tagged Ptr ToxB, whereas in an earlier study (Kim and Strelkov, 2007) strong chlorosis could be induced with 1 μM of the heterologously expressed protein. This difference in activities could reflect the use of different buffers for infiltration of the toxin (12.5 mM Tris HCl (pH 8.0) in this study vs. 20 mM sodium acetate (pH 4.6) in Kim and Strelkov (2007), although Kim and Strelkov (2007) indicated that they tested Ptr ToxB activity in both buffers and found that it was similar. Alternatively, protein estimates may have been influenced by the quantitation methods used, since the Lowry method (Lowry *et al.,* 1951) was the basis for protein quantitation in this study vs. the method of Bradford (1976) in the study by Kim and Strelkov (2007). Regardless of its specific activity, the His-tagged Ptr ToxB used in the current study was bioactive and could induce highly specific chlorosis symptoms on sensitive wheat. As such, it was useful for evaluating the toxin sensitivity of the VarComp population.

Twenty nine of the wheat cultivars included in the current study were tested previously for their sensitivity to Ptr ToxA and Ptr ToxB by Lamari *et al.* (2005). The present results are generally consistent with those of the earlier report (Lamari *et al.,*2005), in which the authors

found that sensitivity to Ptr ToxA in Canadian bread wheat genotypes is more widespread than sensitivity to Ptr ToxB. Nevertheless, some apparent discrepancies were noted. For example, Lamari *et al.* (2005) found that the cultivars '5701 PR', 'Park' and 'Thatcher' were uniformly insensitive to Ptr ToxA, while in the current study these cultivars exhibited a heterogeneous response to the toxin, with a small percentage of the seedlings of each genotype yielding a sensitive reaction. Similarly, most seedlings of 'AC Vista' were insensitive to Ptr ToxA in this study, while the cultivar was reported to be homogenously sensitive to Ptr ToxA by Lamari *et al.* (2005).

The results with respect to sensitivity to Ptr ToxB also were generally similar in both this study and that of Lamari *et al*. (2005). Two exceptions, however, were the reaction of 'Roblin' and '5500 HR'. While Lamari *et al*. (2005) indicated that 'Roblin' was insensitive to Ptr ToxB, this cultivar was found to be sensitive to the toxin in the present experiments. Similarly, the response of '500HR' to Ptr ToxB was heterogeneous in this study, but the cultivar was reported to be homogenously insensitive to the toxin by Lamari *et al.* (2005). One possible explanation for these discrepancies may be the length of time that elapsed between infiltration with Ptr ToxB and scoring for symptom development in the two studies. While in the earlier report (Lamari *et al.,* 2005) host reactions to Ptr ToxB were evaluated 3-4 days following toxin treatment, in the current study the host reactions to Ptr ToxB were assessed over a period of 8 days. In the case of the wheat cultivars '5500 HR' and 'Roblin', chlorosis symptoms did not become clear until 5 and 8 days post-infiltration, respectively. This delay in chlorosis symptom expression may reflect a differential sensitivity to this toxin in these two cultivars.

Nine of the 92 sensitive cultivars gave a heterogeneous reaction to infiltration with at least one of the HSTs. Heterogeneous reactions to the Ptr toxins also were reported previously by

other research groups (Lamari and Bernier, 1989b; Riede *et al.,* 1996; Lamari *et al.,* 2005). Interestingly, in the present study a third of the seedlings of 'Columbus' were sensitive to Ptr ToxA, while the rest were insensitive; other studies also have found that 'Columbus' appears to segregate for sensitivity to Ptr ToxA (Lamari and Bernier, 1989b; Lamari *et al.,* 2005; Riede *et al.,* 1996). Indeed, segregation for sensitivity to particular HSTs may explain the apparently heterogeneous reactions of some cultivars. Another possibility that must be considered, however, is heterogeneity or mixing of the seed lots. Given that some of these seed stocks have been maintained for many years, it is possible that they are not completely pure.

The wheat genotypes assessed in this study represent a collection of Canadian cultivars released over the past century. The same general trends were observed, however, with respect to toxin sensitivity regardless of year of cultivar release (1980s or earlier, 1990s, 2000s, 2010s). Sensitivity to Ptr ToxA was most common among the cultivars, followed closely by sensitivity to Ptr ToxC. The widespread sensitivity to Ptr ToxA may explain the predominance of the Ptr ToxA-producing races 1 and 2 of *P. tritici-repentis* in Canada (Lamari *et al.,* 1998; Lamari *et al.,* 2003; Aboukhaddour *et al.,* 2013). While both historical and modern cultivars with sensitivity to Ptr ToxB also were identified, this trait was much less common than sensitivity to the other HSTs. Fungal isolates producing active forms of Ptr ToxB are extremely rare in Canada, with only a single weakly virulent race 5 isolate identified to date from the Prairie Provinces (Lamari *et al.,* 1998; Strelkov *et al.*, 2002). While the majority of cultivars were found to be sensitive to Ptr ToxC, race 3 of *P. tritici-repentis*, which produces only Ptr ToxC, is fairly rare in Canada (Lamari *et al.,* 1998; Aboukhaddour *et al.,* 2013). By contrast, and as noted above, race 1, which produces both Ptr ToxA and Ptr ToxC, is one of the predominant races along with race 2 (Ptr ToxA only). This suggests that the pathogenicity conferred by Ptr ToxA gives a selective

advantage to the fungus that is greater than any advantage provided by Ptr ToxC, and hence the latter likely has been maintained in Canadian populations of *P. tritici-repentis* by its association with Ptr ToxA in race 1 (Strelkov and Lamari, 2003).

Interestingly, Lamari *et al.* (2005) cited unpublished data from L. Lamari (University of Manitoba) that most hexaploid wheat cultivars from western Canada are resistant to race 3 and "are assumed to be insensitive to Ptr ToxC". This is in contrast with the present results in which nearly two-thirds of the cultivars tested were sensitive to spore germination fluids with Ptr ToxC activity. The current study is the first to examine the sensitivity of wheat cultivars to the Ptr ToxC 'toxic principle' found in spore germination fluids of *P. tritici-repentis*. Previous studies on the sensitivity of wheat to Ptr ToxC have relied on inoculation with fungal isolates presumed to produce this toxin, with sensitivity to Ptr ToxC inferred from susceptibility to the fungus. Infiltration with the toxic principle in spore germination fluids is probably a superior method to evaluate Ptr ToxC sensitivity, since it reduces the likelihood that specific interactions will be masked by the infection process itself or by the production of other non-specific phytotoxic molecules by *P. tritici-repentis* (Bouras and Strelkov, 2008). Although the number and names of the cultivars evaluated by L. Lamari in his unpublished data are not known, if some of the cultivars were the same as those included in this study, it is also possible that other genes or loci beyond the Ptr ToxC-sensitivity gene *Tsc1*may be involved in controlling the response of wheat to *P. tritici-repentis*.

In the study of Lamari *et al.* (2005) and in the present study, sensitivity to Ptr ToxA in Canadian wheat cultivars appears to first have been introduced via 'Red Fife'. This sensitivity was passed to 'Marquis' around 1907 and to 'Neepawa' around 1969. From 'Neepawa' and its derivatives, sensitivity to Ptr ToxA was passed to newer cultivars including 'AC Domain'

(1993), ‘Harvest’ (2004), ‘Kane’ (2006), ‘AC Super’ (2001), ‘CDC Go’ (2004), and ‘CDC Abound’ (2007). Sensitivity to Ptr ToxB probably originated in the cultivar ‘Thatcher’ (1935) and was then transferred to ‘Park’ (1963) and ‘Neepawa’ (1969). As was the case with sensitivity to Ptr ToxA, sensitivity to Ptr ToxB was passed from ‘Neepawa’ to other cultivars including ‘Katepwa’ (1981), ‘AC Domain’ (1993), ‘Harvest’ (2002), and ‘CDC Go’ (2004). Additionally, the results of this study suggest that sensitivity to Ptr ToxC is likely to have been introduced to Canadian wheat cultivars from ‘Neepawa’ (1969), from which it could have been transferred to ‘Columbus’ (1980) and more modern cultivars including ‘AC Barrie’ (1994), ‘AC Cadillac’ (1994), ‘AC Elsa’ (1996), ‘Infinity’ (2004), and ‘CDC Alsask’ (2005).

Occasionally, unexpected cultivar reactions were observed in this study, particularly when considering the reactions of the parents. For example, ‘AC Superb’ (2001) was found to be insensitive to Ptr ToxB even though its parents ‘AC Domain’ (in the current study) and ‘Grandin’ (in Lamari *et al.,* 2005) were sensitive to Ptr ToxB. In the earlier assessment by Lamari *et al.* (2005), the reaction of ‘Grandin’ to Ptr ToxB was heterogeneous, and it is possible that insensitive individuals of ‘Grandin’ were crossed with the sensitive parent ‘AC Domain’, resulting in the insensitive progeny. A few studies have suggested that insensitivity or resistance to Ptr ToxB-type chlorosis induced by race 5 of *P. tritici-repentis* is controlled by a single dominant gene (Singh *et al.*, 2008), although most reports indicate that a single recessive gene is responsible for conferring resistance to race 5 and insensitivity to Ptr ToxB (Gamba *et al.,* 1998; Abeysekara *et al.,* 2010; Singh *et al.,* 2010). These discrepancies suggest that host reaction to Ptr ToxB may be influenced by environmental factors and (or) the genetic background of the wheat.

The present study revealed that the majority of CWRS wheat cultivars in the VarComp population are sensitive to at least one HST. The widespread sensitivity to the Ptr toxins in the CWRS wheat class likely results from the intensive back-crossing of a small number of toxin-sensitive parents. Although this back-crossing helped to maintain superior wheat quality, this method of breeding has led to a low genetic diversity in the CWRS wheat class and to a high level of susceptibility to diseases (DePauw *et al.,* 1998). In general, the results of this study are consistent with the widespread susceptibility of Canadian wheat to tan spot disease, since sensitivity to the Ptr toxins is well-correlated with susceptibility to the respective toxin-producing isolates of *P. tritici-repentis*. Nonetheless, the identification of toxin-insensitive wheat cultivars, particularly in the CPS class where five of eight genotypes tested were insensitive to all three toxins, suggests that there is good potential to develop wheat resistant to the prevalent races of the tan spot pathogen. Efforts to enhance tan spot resistance in Canadian wheat will require attention to the Ptr toxin-sensitivity of the parental material.

Table 2-1. Induction of necrosis by His-tagged Ptr ToxA on the sensitive wheat 'Glenlea'.

Concentration (µmol/l)[a]	**Hours after infiltration[b]**		
	24 h	**48 h**	**72 h**
3.5	+	++	+++
1.4	+	++	+++
0.6	+/-	+	++
0.3	-	+	++
0.1	-	+/-	+
0.01	-	-	+/-
0.001	-	-	+/-

[a] His-tagged Ptr ToxA was infiltrated into the leaves of the sensitive wheat 'Glenlea' at the indicated concentrations.

[b]Necrosis was assessed every 24h, with symptom severity denoted as very severe (+++), severe (++), visible necrosis (+), necrosis localized to the center of the infiltrated site (+/-), and absence of necrosis (-).

Table 2-2. Reaction of the 100 wheat cultivars of the VarComp population to heterologously expressed Ptr ToxA, Ptr ToxB and spore germination fluids possessing Ptr ToxC activity.

	Sensitivity[b]				Sensitivity[b]		
Genotype[a]	**ToxA**	**ToxB**	**ToxC**	**Genotype[a]**	**ToxA**	**ToxB**	**ToxC**
Checks				CDC Thrive	+	-	-
Glenlea	+			CDC Utmost	-	-	+
6B662		+		Columbus	+*	-	+
6B365			+	Fieldstar VB	+	-	+
Salamouni	-	-	-	Garnet	+	-	-
CWRS				Glenn	+	+	-
5500HR	+	+*	+	Goodeve VB	+	+	+
5600HR	-	-	+	Harvest	+	+	-
5601HR	+	-	+	Helios	-	-	+
5602HR	+	-	+	Infinity	-	-	+
5603HY	-	-	+	Journey	+	+*	+
5604HR CL	+	-	-	Kane	+	-	+
859CL	+	-	+	Katepwa	+	+	-
AC Abbey	-	+	-	Laura	-	-	+
AC Barrie	-	-	+	Lillian	-	-	+
AC Cadillac	-	-	+	Lovitt	+	-	-
AC Domain	+	+	+	Marquis	+	-	-
AC Elsa	-	-	+	Mckenzie	+	-	+
AC Eatonia	-	+	-	Muchmore	+	-	+
AC Intrepid	+	+	+	Neepawa	+	+	+
AC Splendor	-	+	+	Park	+*	+	+
AC Superb	+	-	+	Prodigy	+	+	-
Alvena	+	-	+	PT559	+	+	-
BW423	-	-	+	PT580	-	-	+
Carberry	+	-	-	Red Fife	+	-	-
CDC Abound	+	-	+	Roblin	-	+	+*
CDC Alsask	-	-	+	Shaw	+	-	-
CDC Bounty	+	-	-	Somerset	+	-	+
CDC Go	+	+	+	Stettler	+	-	+
CDC Imagine	+	-	-	Thatcher	+*	+	-
CDC Kernen	+	-	+	Unity VB	+	-	+
CDC Merlin	-	-	+	Vesper	+	-	-
CDC Osler	+*	-	+	Waskada	+	+	+*
CDC Stanley	-	-	+				
CDC Teal	+	-	+				

(Continued)

Genotype[a]	Sensitivity[b]			Genotype[a]	Sensitivity[b]		
	ToxA	ToxB	ToxC		ToxA	ToxB	ToxC
CPS				**CWES**			
5700PR	-	-	-	Burnside	+	-	+
5701PR	+*	-	+	CDC Rama	+	-	-
5702PR	+	-	-	CDC Walrus	+	-	+*
AC Crystal	-	-	-	CDN Bison	-	-	+
AC Foremost	-	-	-	Glencross VB	+	-	+
AC Taber	-	-	-	Glenlea	+	-	+
AC Vista	+*	-	+	**CWGP**			
HY682	-	-	-	Minnedosa	+	+	+
SY985	-	-	+	NRG003	-	-	+
CWAD				NRG010	-	-	+
AC Avonlea	-	-	-	**CWSWS**			
AC Morse	+	+	-	AC Andrew	+	-	+
AC Navigator	+	-	-	AC Reed	+	-	+
Brigade	-	-	-	Bhishaj	+	+	+
CDC Verona	+	-	+	Sadash	+	-	+
Commander	+	-	+	**CWHWS**			
DT 570	+	-	-	Snowbird	+	-	+
DT780	+	-	-	Snowstar	+	+	+
Enterprise	+	-	-				
Eurostar	-	-	-				
Kyle	+	-	-				
Napoleon	+	-	+				
Strongfield	+	-	-				
Transcend	-	+	-				

[a] CWRS = Canada Western Red Spring; CPS = Canada Prairie Spring; CWAD = Canada Western Amber Durum; CWES = Canada Western Extra Strong; CWGP = Canada Western General Purpose; CWSWS = Canada Western Soft White Spring; CWHWS = Canada Western Hard White Spring.

[b] Reaction to the Ptr toxins: (+) sensitive and (-) insensitive; an asterisk (*) denotes a heterogeneous reaction (some plants sensitive, others insensitive).

Table 2-3. Reaction of the VarComp population to heterologously expressed Ptr ToxA, Ptr ToxB and spore germination fluids possessing Ptr ToxC activity, grouped by wheat class.

Wheat class[a]	Number of tested cultivars/lines	Number of cultivars sensitive to each toxin		
		ToxA	ToxB	ToxC
CWRS	62	43	19	43
CPS	9	3	0	3
CWAD	14	10	2	3
CWES	6	5	0	5
CWGP	3	1	1	3
CWSWS	4	4	1	4
CWHWS	2	2	1	2

[a] CWRS = Canada Western Red Spring; CPS = Canada Prairie Spring; CWAD = Canada Western Amber Durum; CWES = Canada Western Extra Strong; CWGP = Canada Western General Purpose; CWSWS = Canada Western Soft White Spring; CWHWS = Canada Western Hard White Spring.

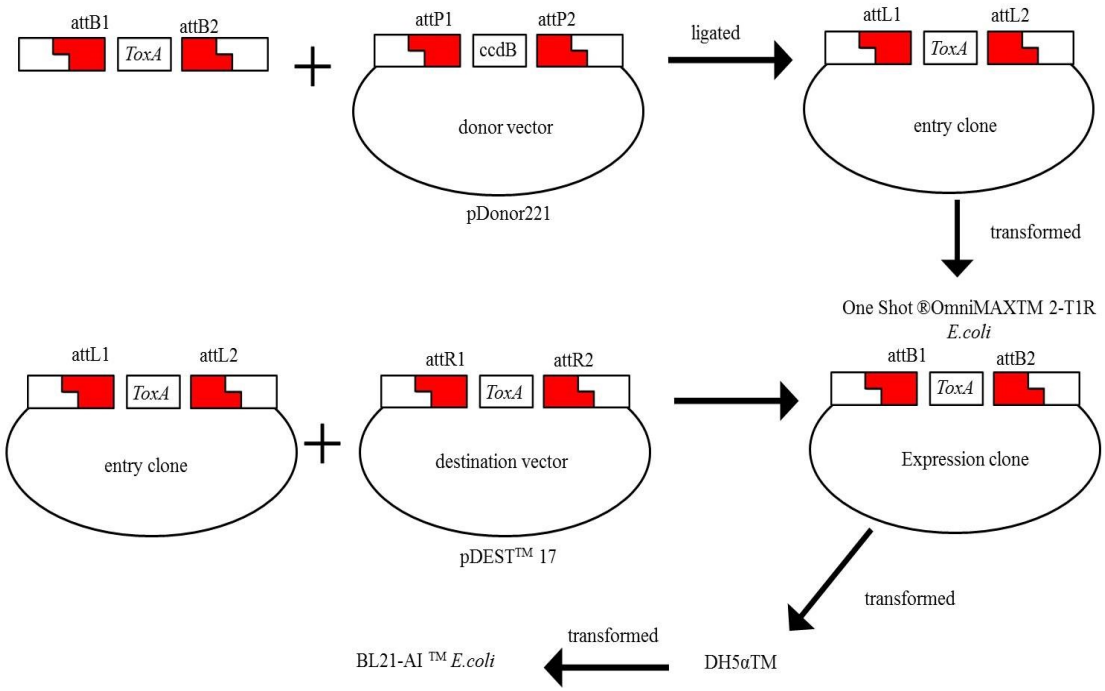

Figure 2-1. Diagrammatic representation of the general procedure involved for the cloning of the ToxA gene (adapted from Invitrogen, Canada).

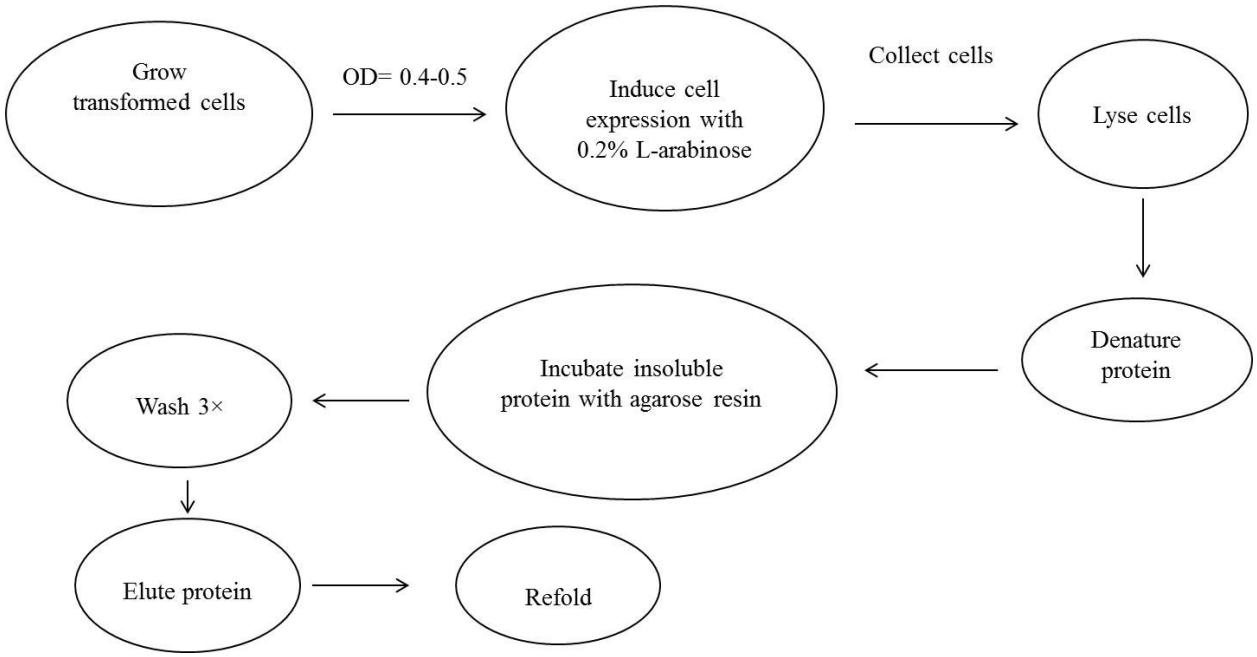

Figure 2-2. Flowchart summarizing the major steps involved in heterologous expression and purification of Ptr ToxA.

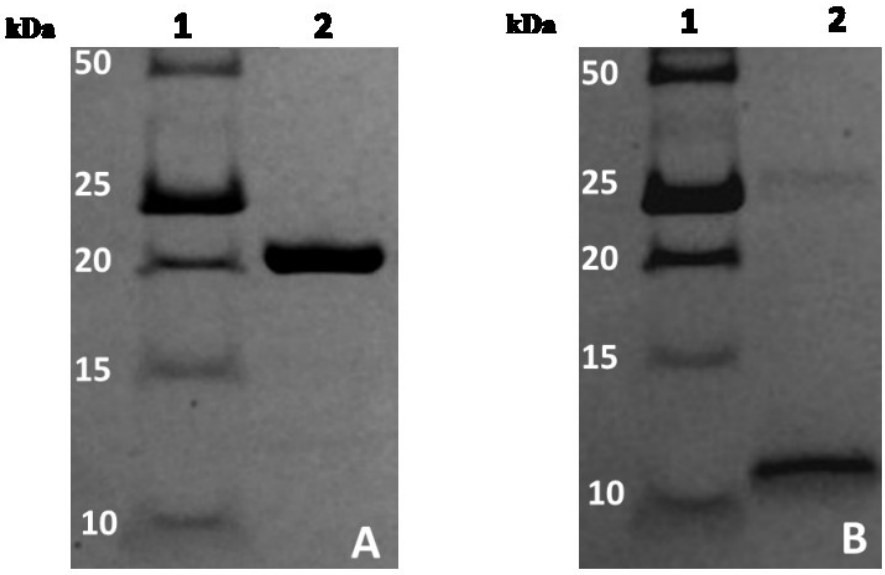

Figure 2-3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of His-tagged Ptr ToxA (A) and His-tagged Ptr ToxB (B). (A) Lane 1, molecular markers (Precision Plus Protein™, Bio-Rad); lane 2, His-tagged Ptr ToxA (approximately 21 kDa). (B) Lane 1, molecular markers (Precision Plus Protein™, Bio-Rad); lane 2, His-tagged Ptr ToxB (approximately 11 kDa).

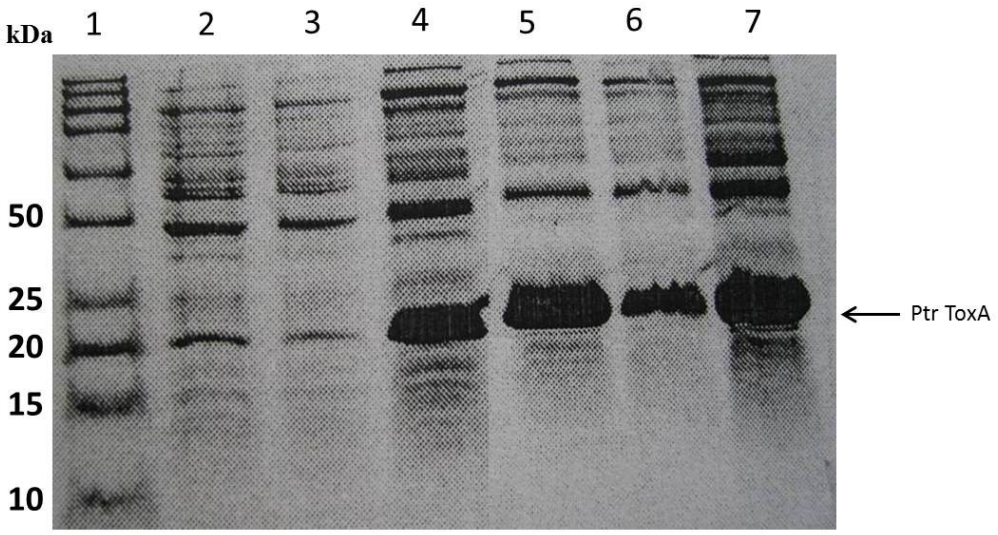

Figure 2-4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of His-tagged Ptr ToxA heterologously expressed in *E. coli*. Lane 1, molecular markers (Precision Plus Protein™, Bio-Rad); the relative abundance of the toxin protein after incubation of bacterial cultures with 0.2% L_arabinose for 0 h (lane 2), 1 h (lane 3), 2 h (lane 4), 3 h (lane 5), 4 h (lane 6), or overnight (lane 7) is shown. The band corresponding to Ptr ToxA is noted.

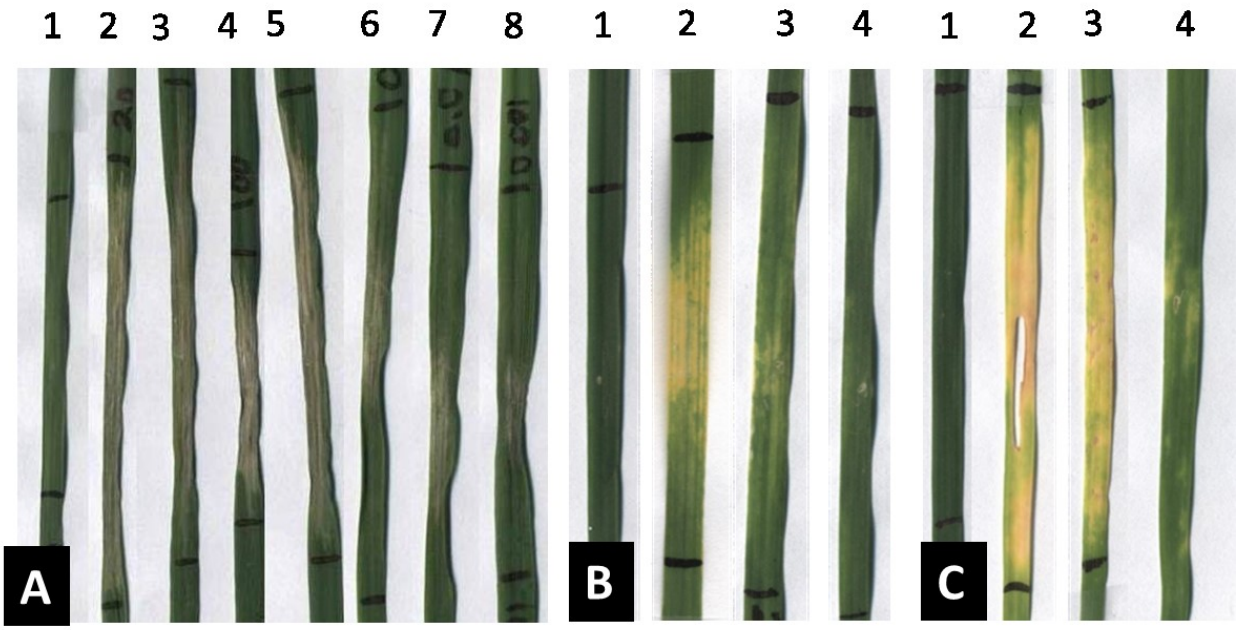

Figure 2-5. Symptoms induced by the Ptr toxins on the reference wheat genotypes 'Salamouni', 'Glenlea', 6B662 and 6B365. (A) Symptom development 48 h after infiltration with His-tagged Ptr ToxA: 'Salamoumi' infiltrated with 3.5 µM Ptr ToxA (1); and 'Glenlea' infiltrated with 3.5 µM (2), 1.4 µM (3), 0.6 µM (4), 0.3 µM (5), 0.1 µM (6), 0.01 µM (7), or 0.001 µM (8) Ptr ToxA. (B) Symptom development 120 h after infiltration with His-tagged Ptr ToxB: 'Salamoumi' infiltrated with 10 µM Ptr ToxB (1); and '6B662' infiltrated with 10 µM (2), 5 µM (3), or 0.5µM (4) Ptr ToxB. (C) Symptom development at two weeks after infiltration with fungal spore germination fluids with Ptr ToxC activity: 'Salamoumi' infiltrated with 1:10 dilutions of spore germination fluids: water (1); and 6B365 infiltrated with 1:10 (2), 1:20 (3), or 1:50 (4) dilutions of spore germination fluids: water.

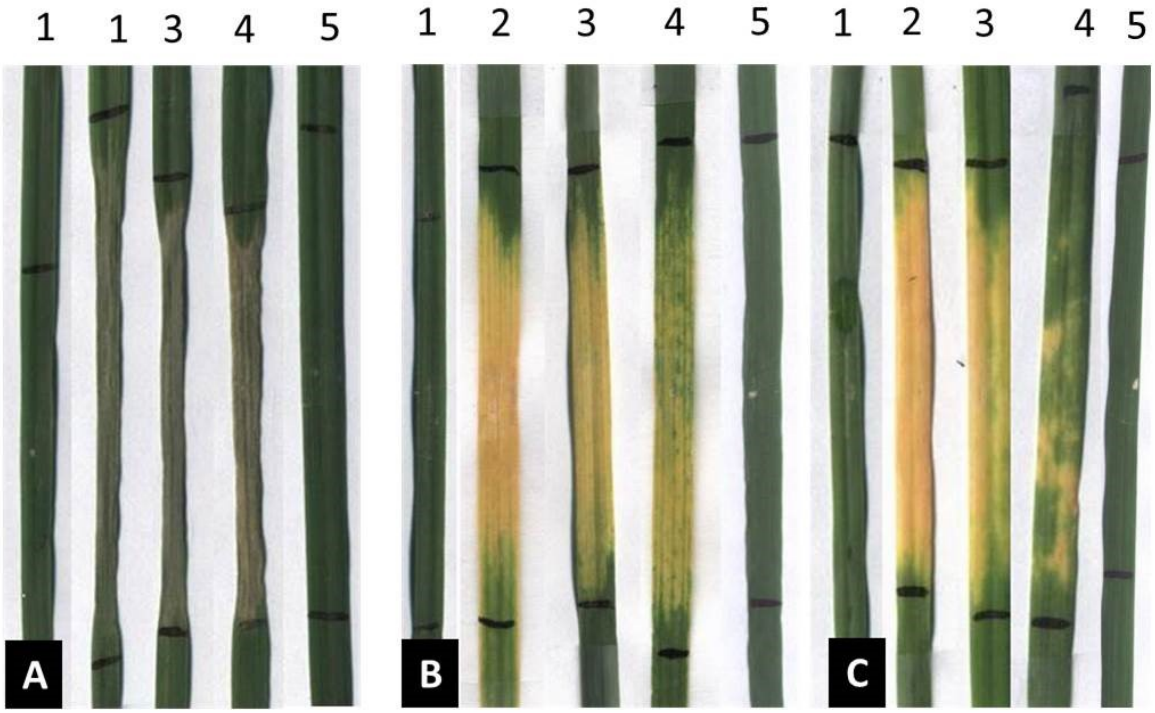

Figure 2-6. The sensitivity of selected wheat cultivars of the 'VarComp' population to His-tagged Ptr ToxA, His-tagged Ptr ToxB and spore germination fluids with Ptr ToxC activity. (A) Sensitivity to Ptr ToxA in 'Salamouni'(1), 'DT 570'(2), 'CDC Teal'(3), 'AC Crystal'(4), and 'Infinity' (5). (B) Sensitivity to Ptr ToxB in 'Salamouni' (1), 'Katepwa' (2), 'Eatonia'(3), 'Journey' (4), and 'Maquis' (5). (C) Sensitivity to Ptr ToxC in 'Salamouni' (1), 'Minnedosa'(2), 'PT 580'(3), 'Burnside' (4), and 'Red Fife'(5).

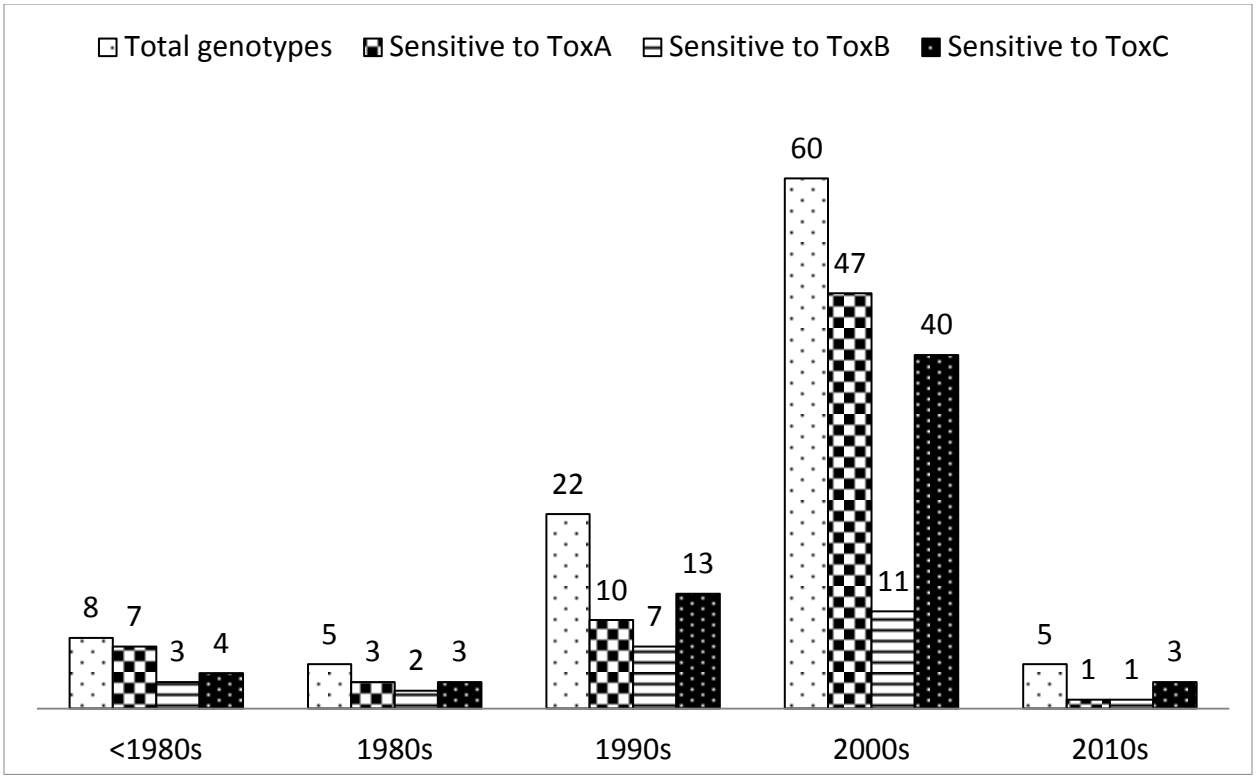

Figure 2-7. The sensitivity of wheat cultivars released prior to the 1980s or in the 1980s, 1990s, 2000s or 2010s, to Ptr ToxA, Ptr ToxB and spore germination fluids with Ptr ToxC activity. The number of cultivars is indicated on the top of each bar.

Chapter 3: Conclusion

Studies on the wheat - *P. tritici-repentis* interaction provide useful information for wheat breeding programs focused on tan spot resistance. The interaction between the host-selective toxins (HSTs) produced by the fungus and the corresponding receptors or targets in the host leads to the development of tan spot disease. Therefore, these HSTs can be considered to be pathogenicity factors for *P. tritici-repentis* (*sensu* Yoder, 1980; Strelkov and Lamari, 2003). Wheat genotypes that are insensitive to the HSTs are potentially resistant to the toxin producing races of the fungus (Lamari *et al.,* 2005; Lamari and Strelkov, 2010). Indeed, cytological studies have revealed that while the initial stages of host infection by *P. tritici-repentis* are similar in HST-sensitive and HST-insensitive wheat, fungal colonization of the host tissues will stop if an isolate does not produce any of the Ptr toxins (Larez *et al.,* 1986; Loughman and Deverall, 1986; Lamari and Bernier, 1989b; Dushnicky *et al.,* 1996). Damage to the host cells in the mesophyll beyond the advancing mycelium results from toxin secretion by *P. tritici-repentis* (Larez *et al.,* 1986; Loughman and Deverall, 1986; Dushnicky *et al.,* 1996). Molecular studies in the host and pathogen also strongly support a role for the Ptr toxins as pathogenicity factors. Ciuffetti *et al.* (1997) found that transformation of the *ToxA* gene encoding Ptr ToxA from a pathogenic to a non-pathogenic isolate of *P. tritici-repentis* was a sufficient condition for virulence on a sensitive wheat genotype. Similarly, silencing of the *ToxB* gene that encodes for Ptr ToxB resulted in significantly reduced virulence on toxin-sensitive wheat, in a manner that was well-correlated with the reduction in the amount of Ptr ToxB produced (Aboukhaddour *et al.,* 2012).

The chlorosis and necrosis symptoms induced by the Ptr toxins are clear, and insensitive or sensitive reactions in wheat easy to distinguish (Tomas and Bockus, 1987). Screening for insensitivity to the HSTs has been conducted together with fungal inoculations to search for

sources of resistance to *P. tritici-repentis*. Insensitivity to Ptr ToxA and resistance to the toxin producing isolates were identified in tetraploid wheat (Chu *et al.,* 2008), in synthetic hexaploid wheat (Xu *et al.,* 2004; Friesen *et al.,* 2008a), in spring wheat from the Northern Great Plains (Singh *et al.,* 2006b), and in wheat germplasm from the International Maize and Wheat Improvement Center (CIMMYT) (Ali *et al.,* 2008). Additionally, a few bread wheat lines were identified with insensitivity to both Ptr ToxA and Ptr ToxB and resistance to the toxin-producing fungal isolates (Lamari *et al.,* 2005). In the current study, a collection of 100 historical and modern Canadian wheat cultivars known as the 'VarComp' population was assessed for sensitivity to all three HSTs produced by *P. tritici-repentis*. The aim of the research was to provide detailed information on the sensitivity to these toxins in Canadian wheat cultivars representing a wide variety of classes and years. This also was the first time that a comprehensive assessment for sensitivity to Ptr ToxC was carried out.

Ptr ToxA was heterologously expressed in *E. coli*, with the resulting protein purified and characterized for activity. The His-tagged Ptr ToxA could be expressed in high amounts and induced necrosis in a highly specific manner. Similarly, Ptr ToxB that had been previously cloned and transformed into *E. coli* (Kim and Strelkov, 2007) also was expressed and purified. Fungal spore germination fluids possessing Ptr ToxC activity were produced by incubation of the conidia on detached leaf segments. Unlike Ptr ToxA and Ptr ToxB, Ptr ToxC has not been fully purified and its chemical nature is not completely understood (Effertz *et al.,* 2002). While, additional research is need to characterize this toxin, the Ptr ToxC activity associated with spore germination fluids in this study represents the first time that the toxic principle has been produced *in vitro*. The Ptr ToxA, Ptr ToxB and Ptr ToxC preparations that were generated as part of this research served as tools to screen the 100 wheat cultivars of the VarComp population for

sensitivity to these toxins. Wheat seedlings were scored for the presence (+) or absence (-) of necrosis or chlorosis at the toxin-infiltration sites, with the reactions serving as an indication of the sensitivity or insensitivity of a particular genotype to each HST.

The vast majority (92%) of the wheat cultivars tested was sensitive to at least one HST, with most genotypes sensitive to Ptr ToxA (68%) and Ptr ToxC (63%). These findings highlight the threat that tan spot poses to the cultivation of Canadian wheat, since races 1 and 2 of *P. tritici-repentis*, which produce Ptr ToxA + Ptr ToxC and Ptr ToxA, respectively, are the most prevalent races in Canada (Aboukhaddour *et al.,* 2013; Lamari *et al.,* 1998; Lamari *et al.,* 2003). While less common, sensitivity to Ptr ToxB also was identified in 24% of the cultivars. By contrast, eight cultivars were found to be insensitive to all of the Ptr toxins, suggesting that these cultivars also would be resistant to the toxin producing races and may represent good sources of tan spot resistance. Twenty nine of the 100 wheat genotypes tested in this study also were tested previously for sensitivity to Ptr ToxA and Ptr ToxB by Lamari *et al.,* (2005). The results of the present study were similar to those reported in the earlier analysis (Lamari *et al.,* 2005) with a few of exceptions. For instance, the cultivar 'Roblin' was found to be sensitive to Ptr ToxB in this thesis, but was described as insensitive by Lamari *et al.* (2005). As noted in Chapter 2, this discrepancy may reflect the fact that the reaction to Ptr ToxB was evaluated after 3-4 days in the study by Lamari *et al.* (2005), versus after 8 days in the present report. This suggests that the evaluation period to assess wheat for sensitivity to Ptr ToxB should be extended to 8 days after infiltration.

Sensitivity to the three Ptr toxins is known to be controlled by single dominant genes, with one gene for each toxin (Anderson *et al.,* 1999; Faris *et al.,* 1996; Gamba *et al.,* 1998). Therefore, we can predict that wheat cultivars and lines that are sensitive to Ptr ToxA, Ptr ToxB

and Ptr ToxC carry the sensitivity genes *Tsn1*, *Tsc2* and *Tsc1*, respectively. For example, 'Lilian' was found to be sensitive only to Ptr ToxC and is predicted to carry *Tsc1*. 'CDC Kernen' was sensitive to Ptr ToxA and Ptr ToxC and so may carry both *Tsn1* and *Tsc1*. The current findings may be useful in future studies to develop markers for maker-assisted selection, since markers for the toxin-sensitivity genes would be helpful for eliminating these genes from wheat germplasm. Additionally, these markers could be used to avoid the inadvertent introduction of novel sources of sensitivity from breeding materials.

Lamari and Bernier (1991) studied the genetics of the inheritance of toxin sensitivity in 'Glenlea', 6B365, 'Salamouni' by making crosses between these genotypes, and found that Ptr ToxA insensitivity in the host is strongly associated with resistance to race 1. In addition, Chu *et al.* (2008) suggested that insensitivity to Ptr ToxA increased resistance to tan spot in wild emmer wheat. Lamari *et al*. (2005) found a 100% correlation between insensitivity/sensitivity to Ptr ToxA and Ptr ToxB and resistance/susceptibility to races 2 and 5 of *P. tritici-repentis* in Canadian Western Red Spring wheat. By contrast, Friesen *et al.* (2003) indicated that the insensitivity to Ptr ToxA in F2-derived-F6 recombination inbred lines was not always correlated with resistance to a Ptr ToxA-producing (race 2) isolate, and that insensitivity to Ptr ToxA contributed only 24.4% of the resistance to race 2 (Friesen *et al.,* 2003). Faris and Friesen (2005) also found that race non-specific resistance genes were located on the wheat chromosomes 1B and 3B in a wheat population segregating for Ptr ToxA sensitivity. There have been a few other reports stating that some wheat cultivars that are insensitive to Ptr ToxA are nonetheless susceptible to spore suspensions of race 2 isolate 86-124 (Riede *et al.,* 1996; Singh *et al.,* 2006a), indicating that additional factors present in the spore suspension may assist with the development of tan spot necrosis. Indeed, in the present study, nearly two-thirds of the tested wheat genotypes

were sensitive to Ptr ToxC, while previous unpublished data from L. Lamari (cited in Lamari *et al*., 2005) indicated that most Canadian hexaploid wheat cultivars are resistant to race 3 (Ptr ToxC producer). Therefore, while data on the sensitivity/insensitivity of wheat genotypes to the Ptr toxins may provide a good indication of their susceptibility/resistance to the races of *P. tritici-repentis*, it may be worthwhile to validate the current results by inoculation of the VarComp population with isolates of the fungus representing race 2 (Ptr ToxA producer), race 3 (Ptr ToxC producer), and race 5 (Ptr ToxB producer).

Breeding programs based on limited genetic backgrounds have unintentionally introduced and maintained the sensitivity to tan spot in Canadian wheat (Lamari *et al.,* 2005). It is interesting to note that nearly one-quarter of the cultivars tested in this study were sensitive to Ptr ToxB. While the absence of Ptr ToxB producing isolates in Canada has been fortuitous (Strelkov *et al.,* 2002; Aboukhaddour *et al.,* 2013), the Ptr ToxB-sensitivity of many of the cultivars of the VarComp population suggests a serious threat to Canadian wheat if Ptr ToxB producing isolates were to be introduced to this country.

At least two of the Ptr toxins have been found to occur across species boundaries. *P. tritici-repentis* appears to have acquired the *ToxA* gene from *Stagonospora nodorum* by horizontal gene transfer as recently as 1941 (Friesen *et al.*, 2006). The form of *ToxA* in *S. nodorum*, which is another common foliar pathogen of wheat, encodes a HST that is very similar to Ptr ToxA and has been designated as Sn ToxA to denote its *S. nodurum* origin. Eleven haplotypes of *ToxA* were found in *S. nodurum*, while only one haplotype of *ToxA* has been identified in *P. tritici-repentis* (Friesen *et al.,* 2006). Similarly, in recent years, *ToxB*-like sequences have been reported from *Pyrenophora bromi* and other ascomycetes (Andrie *et al.,* 2008), suggesting that this toxin also may mediate host-pathogen interactions in a number of

pathosystems. Indeed, the fact that a race 5 isolate of *P. tritici-repentis* can cause chlorosis symptoms on barley, similar to those that it causes on wheat (Kim, 2012), indicates that information on toxin sensitivity/insensitivity may be important not only for the management of tan spot, but also for understanding of other related pathosystems.

References

Abeysekara, N.S., Friesen, T.L., Liu, Z.H., McClean, P.E., and Faris, J.D. 2010. Marker development and saturation mapping of the tan spot Ptr ToxB sensitivity locus *Tsc2* in hexaploid wheat. Plant Genome 3:179–189.

Aboukhaddour, R., Cloutier, S., Balance, G.M., and Lamari, L.2009. Genome characterization of *Pyrenophora tritici-repentis* isolates reveals high plasticity and independent chromosomal location of *ToxA* and *ToxB*. Molecular Plant Pathology 10:201-212.

Aboukhaddour, R., Kim, Y.M., and Strelkov, S.E. 2012. RNA-mediated gene silencing of *ToxB* in *Pyrenophora tritici-repentis*. Molecular Plant Pathology 13: 318-326.

Aboukhaddour, R., Turkington, T.K., and Strelkov, S.E. 2013. Race structure of *Pyrenophora triciti-repentis* (tan spot of wheat) in Alberta, Canada. Canadian Journal of Plant Pathology 35: 256-268.

Ali, S., Mergoum, M., Adhikari, T. B., Singh, P. K., and McMullen, M. P. 2008. Resistance to multiple leaf spot diseases in wheat. Euphytica 159:167-179.

Amaike, S., Ozga, J.A., Basu U., and Strelkov, S.E. 2008. Quantifiction of *ToxB* gene expression and formation of appressoria by isolates of *Pyrenophora tritici-repentis* differing in pathogenicity. Plant Pathology 57: 623-633.

Anderson, J.A., Effertz, R.J., Faris, J.D., Francl, L.J., Meinhardt, S.W., and Gill, B.S. 1999. Genetic analysis of sensitivity to a *Pyrenophora tritici-repentis* necrosis-inducing toxin in durum and common wheat. Phytopathology 89**:** 293–297.

Andrie, R.M., and Ciuffetti, L.M. 2011. *Pyrenophora bromi*, causal agent of brownspot of bromegrass, expresses a gene encoding a protein with homology and similar activity to Ptr ToxB, a host-selective toxin of wheat. Molecular Plant-Microbe Interactions 24:359-36

Andrie, R.M., Schoch, C.L., Hedges, R., Spatafora, J.W., and Ciuffetti, L.M. 2008. Homologs of ToxB, a host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome of sister-species *Pyrenophora bromi* and other members of the Ascomycota. Fungal Genetics and Biology 45: 363-377.

Bailey, K.L., Gossen, B.D., Lafond, G.P., Watson, P.R., and Derksen, D.A. 2001. Effect of tillage and crop rotation on root and foliar diseases of wheat and pea in Saskatchewan from 1991 to 1998: Univariate and multivariate analyses. Canadian Journal of Plant Science
81: 789-803.

Bailey, K.L., Johnston, A.M., Kutcher, H.R., Gossen, B.D., and Morrall, A.A. 2000. Managing crop losses from diseases with fungicides, rotation, and tillage in the Saskatchewan Parkland. Canadian Journal of Plant Science 80: 169-175.

Bailey, K.L., Mortensen, K., and Lafond, G.P. 1992. Effects of tillage systems and crop rotations on root and foliar diseases of wheat, flax, and peas in Saskatchewan. Canadian Journal of Plant Science 72: 583-591.

Ballance, G. M., Bernier, C.C., and Lamari, L. 1989. Purification and characterization of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. Physiological and Molecular Plant Pathology 35: 203-213.

Ballance, G.M., Lamari, L., Kowatsch, R., and Bernier, C.C. 1996. Cloning, expressionand occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis.* Molecular Plant Pathology On-line:http://www.bspp.org.uk/mppol/1996/1209ballance.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein dye-binding. Analytical Biochemistry 72: 248-254.

Barrus, M.F. 1942. Yellow - spot disease of wheat in the New York State. Plant Disease Reporter 26: 246.

Bockus, W.W. 1998. Control strategies for stubble-borne pathogens of wheat. Canadian Journal of Plant Pathology 20: 371-375.

Bockus, W.W., and Shroyer, J.P. 1998. The impact of reduced tillage on soilborne plant pathogens. Annual Review of Phytopathology 36: 485-500.

Bouras, N., and Strelkov, S.E. 2008. The anthraquinone catenarin is phytotoxic and produced in leaves and kernels of wheat infected by *Pyrenophora tritici-repentis*. Physiological and Molecular Plant Pathology 72: 87-95.

Bouras, N., Kim, Y.M., and Strelkov, S.E. 2009. Influence of water activity and temperature on growth and mycotoxin production by isolates of *Pyrenophora tritici-repentis* from wheat. International Journal of Food Microbiology 131: 251–255.

Cheong, J., Wallwork H., and Williams, K.J. 2004. Identification of a major QTL for yellow leaf spot resistance in the wheat varieties Brookton and Cranbook. Australian Journal of Agricultural Research 55:315–319.

Chu, C.G., Nevo, E., Friesen, T.L., Xu, S.S., and Faris, J.D. 2008. Seedling resistance to tan spot and Stagonospora nodorum leaf blotch in wild emmer wheat (*Triticum dicoccoides)*. Plant Disease 92: 1229-1236.

Ciuffetti, L.M., Betts, M.F., Martinez, J.P., Manning, V.A., and Pandelova, I. 2010. Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*-wheat interaction. New Phytologist 187: 911-919.

Ciuffetti, L.M., Francl, L.J., Ballance, G.M., Bockus, W.W., Lamari, L., Meinhardt, S.W., and Rasmussen, J.B. 1998. Standardization of toxin nomenclature in the *Pyrenophora tritici-repentis*/wheat interaction. Canadian Journal of Plant Pathology 20: 421-424.

Ciuffetti, L.M., Tuori, R.P., and Gaventa, J.M. 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. The Plant Cell 9: 135-144.

Colson, E.S., Platz G.J., and Usher, T.R. 2003. Fungicidal control of *Pyrenophora tritici-repentis* in wheat. Australian Plant Pathology 32: 241-246.

Conners, I.L. 1939. Nineteenth annual report of the Canadian Plant Disease Survey 19: 12-14.

De Wolf, E.D., Effertz, R.J., Ali, S., and Francl, L.J. 1998. Vistas of tan spot research. Canadian Journal of Plant Pathology 20: 349-370.

Depauw, R.M., Boughton, G.R., and Knott, D.R. 1995. Hard red spring wheat. Pages 5-35 in: Harvest of Gold- The History of Field Crop Breeding in Canada. A. E. Slinkard and D. R. Knott, eds. University Extension Press, University of Saskatchewan, Canada.

DePauw, R., and Hunt, T. 2001. Canadian Wheat Pool. Pages 479-515 in: The World Wheat Book, A History of Wheat Breeding. A.P. Bonjean and W.J. Angus, eds. Lavoisier Publishing, Paris.

DePauw, R.M., Clarke, J.M., McCaig, T.N., and Townley-Smith, F.T. 1998. Opportunities for improvement of western Canadian protein concentration, grain yield and quality through plant breeding. Pages 75-93 in: Wheat Production and Marketing. University of Saskatchewan, Saskatoon, SK.

Dhingra, O.D., and Sinclair, J.B. 1998. Basic plant pathology methods, CRC Press, Boca Raton.

Dickenson, F.L. 1976. Prairie wheat. Three centuries of wheat varieties in western Canada. Canadian Grains Council, Winnipeg, MB.

Diedicke, H. 1902. Ueber den Zussammenhang Zwischen *Pleospora* - und *Helminthosporium* - Arten. Centrablatt fur Bakteriologie und Parasitenkunde Jena, Abt. 9: 317-329.

Diedicke, H. 1904. Ueber den zusammenhang zwischen *Pleospora* und *Helminthosporium* arten. Zeitschirft fur Pflanzenkrankheiten 11: 317-329.

Dreschler, C. 1923. Some graminicolous species of *Helminthosporium*.I. Journal of Agricultural Research 24: 641-740.

Dushnicky, L. G., MacGregor, A. W., Summer, M. J., and Ballance, G. M. 1996. Penetration and infection of susceptible and resistant wheat cultivars by a necrosis toxin-producing isolate of *Pyrenophora tritici-repentis*. Canadian Journal of Plant Pathology 18: 392-402.

Dushnicky, L.G., Ballance, G.M., Sumner, M.J., and MacGregor, A.W. 1998a. Detection of infection and host responses in susceptible and resistant wheat cultivars to a toxin producing isolate of *Pyrenophora tritici-repentis.* Canadian Journal of Plant Pathology 20: 19-27.

Dushnicky, L.G., Ballance, G.M., Sumner, M.J., and MacGregor, A.W. 1998b. The role of lignification as a resistance mechanism in wheat to a toxin-producing isolate of *Pyrenophora tritici-repentis.* Canadian Journal of Plant Pathology 20: 35-47.

Effertz, R.J., Meinhardt, S.W., Anderson, J.A., Jordahl, J.G., and Francl, L.J. 2002. Identification of a chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. Phytopathology 92: 527-533.

Effertz, R.J., Anderson, J.A., and Francl, L.J. 2001. Restriction fragment length polymorphism mapping of resistance to two races *of Pyrenophora tritici-repentis* in adult and seedling wheat. Phytopathology 91: 572- 578.

Faris J.D., Anderson, J.A., Francl, L.J., and Jordahl, J.G. 1997. RFLP mapping of resistance to chlorosis induction by *Pyrenophora tritici-repentis* in wheat. Theoretical and Applied Genetics 94:98–103.

Faris, J. D., and Friesen, T. L. 2005. Identification of quantitative trait loci for race-nonspecific resistance to tan spot in wheat. Theoretical and Applied Genetics 111: 386-392.

Faris, J.D., Anderson, J.A., Francl, L.J., and Jordahl, J.G. 1996. Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis.* Phytopathology 86: 459-463.

Faris, J.D., Liu, Z., and Xu, S.S. 2013. Genetics of tan spot resistance in wheat. Theoretical and Applied Genetics 126:2197-2217.

Fernandez, M.R., Clarke, J.M., De Pauw, R.M., and Lefkovitch, L.P. 1997. Emergence and growth of durum wheat derived from red smudge-infected seed. Crop Science 37: 510-514.

Flor, H. 1971. Current status of gene-for-gene concept. Annual Review of Phytopathology 9: 275–296.

Francl, L.J. 1998. Genesis and liberation of conidia of *Pyrenophora tritici- repentis*. Canadian Journal of Plant Pathology 20: 387-393.

Francl, L.J. and Jordhal, J.G. 1992. Seed symptomology of durum wheat. In Proceedings of the Second International Tan Spot Workshop.25-26 June 1992, Fargo, N.D. Edited by L.J. Francl, J.M. Krupinsky, and M.P. McMullen. North Dakota State University, Fargo, N.D. Page 61-64.

Fraser, J. G., and Whiteside, A. G. O. 1956. Handbook of CanadianSpring Wheat Varieties. Canada Department of Agriculture, publicationno. 538. Ottawa, ON, Canada.

Friesen, T.L., AH, S., Stack, R.W., Francl, L.J., and Rasmussen, J.B. 2003a. Rapid and efficient production of the *Pyrenophora tritici-repentis* teleomorph. Canadian Journal of Botany 81: 890-895.

Friesen, T.L., Francl, L.J., Rasmussen, J.B., Ali, S., and Kianian, S. 2003b. Role of host sensitivity to Ptr ToxA in development of tan spot of wheat. Phytopathology 93: 397-401.

Friesen, T. L., Harris, M. O., and Xu, S. S. 2008a. Stem rust, tan spot, stagonospora nodorum blotch, and hessian fly resistance in langdon durum-aegilops tauschii synthetic hexaploid wheat lines. Crop Science 48: 1062-1070.

Friesen, T.L., Zhang, Z., Solomon, P.S., Oliver, R.P., and Faris, J.D. 2008b. Characterisation of the interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. *Plant Physiology* 146**:** 682–693.

Friesen, T. L., Solomon, P. S., Rasmussen, J. B., Oliver, R. P., McDonald, B. A., Faris, J. D., Liu, Z., Stukenbrock, E. H., Ling, H., and Meinhardt, S. 2006. Emergence of a new disease as a result of interspecific virulence gene transfer. Nature Genetics 38: 953-956.

Friesen, T.L., and Faris, J.D. 2004. Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to Ptr ToxB in wheat. Theoretical and Applied Genetics 109:464–471.

Gamba, F.M., Strelkov, S.E., and Lamari L. 2012. Virulence of *Pyrenophora tritici-repentis* in the southern cone region of south America. Canadian Journal of Plant Pathology 34: 545–550.

Gamba, F.M., and Lamari, L. 1998. Mendelian inheritance of resistance to tan spot (*Pyrenophora tritici-repentis*) in selected genotypes of durum wheat (*Triticum turgidum*). Canadian Journal of Plant Pathology 20:408–414.

Gamba, F.M., Lamari, L., and Brule-Babel, A.L. 1998. Inheritance of race-specific necrotic and chlorotic reactions induced by *Pyrenophora tritici-repentis* in hexaploid wheats. Canadian Journal of Plant Pathology 20: 401-407.

Hagborg, W.A.F. 1970. A device for injecting solutions and suspensions into thin leaves of plants. Canadian Journal of Botany 48:1135-1136.

Hosford, R.M. Jr., Larez, C.R., and Hammond, J.J. 1987. Interaction of wet period and temperature on *Pyrenophora tritici-repentis* infection and development in wheat of differing resistance. Phytopathology 77: 1021-1027.

Hosford, R.M., Jr. 1972. Propagules of *Pyrenophora trichostoma.* Phytopathology 62: 627-629.

Hosford, R.M.Jr. 1971. A form of *Pyrenophora trichostoma* pathogenic to wheat and other grasses. Phytopatology 62: 28-32.

Hosford, R.M.Jr. 1982. Tan spot - Developing knowledge 1902 - 1981. In Tan Spot of Wheat and Related Diseases Workshop. July. 1981. Fargo, N.D. Edited by Hosford, R.M.Jr. North Dakota Agricultural Experimental Station, North Dakota State University, Fargo, N.D. pp: 1-24

Johnson, A.G. 1942. *Helminthosporium tritici-vulgaris* on wheat in Maryland. The Plant Disease Reporter 26: 246-247.

Jørgensen, L.N., and Olsen, L.V. 2007. Control of tan spot (*Drechslera tritici-repentis*) using cultivar resistance, tillage methods and fungicides. Crop Protection 26: 1606–1616.

Kim, Y.M. 2012. Studies on the role and mechanism of action of Ptr ToxB from *Pyrenophora tritici-repentis*. PhD thesis. Department of Agricultural, Food, and Nutritional Science, University of Alberta.

Kim, Y.M., and Strelkov, S.E. 2007. Hetrologous expression and activity of Ptr ToxB from virulent and avirulent isolates of *Pyrenophore tritici-repentis*. Canadian Journal of Plant Pathology 29: 232-242.

Kim, Y.M, Bouras, N, Kav, N.N, and Strelkov, S.E. 2010. Inhibition of photosynthesis and modification of the wheat leaf proteome by Ptr ToxB: a host-specific toxin from the fungal pathogen *Pyrenophora tritici-repentis*. Proteomics: 10:2911–2926.

Krupinky, J.M. 1982. Observations on the host range of isolates *of Pyrenophora tritici-repentis.* Canadian Journal of Plant Pathology 4: 42-46.

Kwon, C.Y., Rasmussen, J.B., Francl, L.J., and Meinhardt, S.W. 1996. A quantitative bioassay for necrosis toxin from *Pyrenphora tritici-repentis* based on electrolyte leakage. Phytopathology 86: 1360-1363.

Lamari, L., and Bernier, C.C. 1989a. Evaluation of wheat lines and cultivars to tan spot *(Pyrenophora tritici-repentis)* based on lesion type. Canadian Journal of Plant Pathology 11:49-56.

Lamari L., and Bernier C.C., 1989b. Virulence of isolates of *Pyrenophora tritici-repentis* on 11 wheat cultivars and cytology of the differential host reactions. Canadian Journal of Plant Pathology 11: 284-290.

Lamari, L., and Bernier, C.C. 1989c. Toxin of *Pyrenophora tritici-repentis:* Host specificity, significance in disease, and inheritance of host reaction. Phytopathology 79:740-744.

Lamari, L., and Bernier, C.C. 1991. Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. Phytopathology 81:1092–1095.

Lamari, L., and Strelkov, S.E. 2010. The wheat/*Pyrenophire tritici-repentis* reaction: progress towards an understanding of tan spot disease. Canadian Journal of Plant Pathology 32: 4-10.

Lamari, L., McCallum, B. D., and Depauw, R. M. 2005. Forensic pathology of Canadian bread wheat: The case of tan spot. Phytopathology 95: 144-152.

Lamari, L., Sayoud, R., Boulif, M., and Bernier, C.C. 1995. Identification of a new race of *Pyrenophora tritici-repentis:* implications for the current pathotype classification system. Canadian Journal of Plant Pathology 17: 312-318.

Lamari, L., Strelkov, S.E., Yahyaoui, A., Orabi, J., and Smith, R.B. 2003. The identification of two new races *of Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. Phytopathology 93:391-396.

Lamari, L., Tekauz, A., and Gilbert, J. 1998. Race differentiation in *Pyrenophora tritici-repentis* and survey of physiologic variation in western Canada. Canadian Journal of Plant Pathology 20: 396-400.

Landy, A. 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annual Reviews in Biochemistry 58: 913–941.

Larez, C.R., Hosford, R.M., Jr., and Freeman, T.P. 1986. Infection of wheat and oats by *Pyrenphora tritici-repentis* and initial characterization of resistance. Phytopathology 76: 931-938.

Liberman, D.F., Schaefer, F.L., Fink, R.C., Ramgopal, M., Ghosh, A.C., and Mulcahy, R. 1980. Mutagenicity of islandicin and chrysophanol in the Salmonella/microsome system. Applied and Environmental Microbiology 40: 476–479.

Lichter, A., Gaventa, J.M., and Ciuffetti, L.M. 2002. Chromosome-based molecular characterization of pathogenic and non-pathogenic wheat isolates of *Pyrenophora triticirepentis.* Fungal Genetics and Biology 37: 180-189.

Loughman, R and Deverall, B.J. 1986. Experiments with excised lesions from wheat leaves infected by *Pyrenophora tritici-repentis*. Australasian Journal of Plant Pathology 18: 90-93.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry 193: 265-275.

Manning, V.A., and Ciuffetti, L.M. 2005. Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import in wheat mesophyll cells. The Plant Cell 17: 3203-3212.

Manning, V.A., Ciuffetti, L.M., and Hardison, L.K. 2007. Ptr ToxA Interacts with a chloroplast-localized protein. Molecular Plant-Microbe Interactions 20: 168-177.

Manning, V.A., Hamilton, S.M., Karplus, P.A., and Ciuffetti, L.M. 2008. The Arg-Gly-Asp-containing, solvent-exposed loop of Ptr ToxA is required for internalization. Molecular Plant-Microbe Interactions 21: 315–325.

Manning, V.A., Wolpert, T.J., Ciuffetti, L.M., Chu, A.L., and Steeves, J.E. 2009. A Host-Selective Toxin of *Pyrenophora tritici-repentis*, Ptr ToxA, Induces photosystem changes and reactive oxygen species accumulation in sensitive wheat. Molecular Plant-Microbe Interactions 22: 665-676.

Martinez, J.P., Oesch, N.W., and Ciuffetti, L.M. 2004. Characterization of the multiplecopy host-selective toxin gene, *ToxB,* in pathogenic and nonpathogenic isolates of *Pyrenphora tritici-repentis*. Molecular Plant-Microbe Interactions 17: 467-474.

McCallum, B.D., and DePauw, R.M. 2008. A review of wheat cultivars grown in the Canadian prairies. Canadian Journal of plant pathology 88:649-677.

Meinhardt, S.W., Cheng W., Kwon, C.Y., Donohue, C.M., and Rasmussen, J.B. 2002. Role of the Arginyl-Glycyl-Aspartic motif in the action of Ptr ToxA produced by *Pyrenophora tritici-repentis*. Plant Physiology 130:1545–1551.

Mitra, M. 1931. Report of the imperial mycologist. Review of Applied Mycology 10:436.

Mitra, M. 1934. A leaf spot disease of wheat caused by *Helminthosporium triticirepentis* Died. Review of Applied Mycology 14: 90.

Morrall, R.A.A., and Howard, R.J. 1975. The epidemiology of leaf spot disease in a native prairie. II. Airborne spore populations *of Pyrenophora tritici-repentis.* Canadian Journal of Botany 53: 2345-2353.

Morrison, J. W. 1960. Marquis wheat - a triumph of scientific endeavor. Agricultural History 34: 182-188.

Newman, L. H. 1928. The history and present status of wheat production in Canada. Dominion of Canada, Department of Agriculture, Ottawa, ON. Pamphlet No. 89.

Nisicado, Y. 1928. Studies on the Helminthosporiose of Japanese graminaceous plants. Spec.rep. Ohara Inst. Agr. Res. IV. (In Japanese with English descriptions of new species).

Pandelova, I., Betts, M.F., Manning, V.A., Wilhelm, L.J., and Mockler, T.C. 2009. Analysis of transcriptome changes induced by Ptr ToxA in wheat provides insights into the mechanisms of plant susceptibility. Molecular Plant-Microbe Interactions 22: 1067–1083.

Pandelova, I., Figueroa, M., Wilhelm, L.J., Manning, V.A., Mankaney, A.N., Mockle, T.C., and Ciuffetti, L.M. 2012. Host selective toxins of *Pyrenophora tritici-repentis* induce common responses associated with host susceptibility. PLoS ONE 7: e40204.doi: 10.1371/journal. pone.0040240.

Pringle, R.B., and Scheffer, R.P. 1964. Host-specific plant toxins. Annual Review of Phytopathology 2:133-156.

Rees, R.G., and Platz, G.J. 1989. Effectiveness of incomplete resistance to *Pyrenophora tritici-repentis* in wheat. Australian Journal of Agricultural Research 40: 43-48.

Rees, R.G., and Platz, G.J. 1990. Sources of resistance to *Pyrenophora tritici-repentis* in bread wheat. Euphytica 45: 56-69.

Rees, R.G., and Platz, G.J. 1979. The occurrence and control of yellow spot of wheat in north-eastern Australia. Australian Journal of Experimental Agriculture and Animal Husbandry 19: 369-372.

Rees, R.G., Platz, G.J., and Mayer, R.J. 1982. Yield losses in wheat from yellow spot: comparison of estimates derived from single tillers and plots. Australian Journal of Agricultural Research 33: 899-908.

Rees, R.G., Platz, G.J., and Mayer, R.J. 1988. Susceptibility of Australian wheats to *Pyrenophora tritici-repentis.* Australian Journal of Agricultural Research 39: 141-151.

Rees, R.G., and Platz, G.J. 1992. Tan sot and its control – some Autralian experiences. In: Advances in Tan Spot Research: Proceedings of the 2nd International Tan Spot Workshop. 25-26 June 1992. Fargo, N.D. Edited by L.J. Francl, J.M. Krupinsky, and M.P. McMullen. North Dakota Agricultural Experiemental Station, North Dakota State University, Fargo, N.D. pp. 1-9.

Riede, C. R., Jordahl, J. G., Meinhardt, S. W., Francl, L. J., and Anderson, J. A. 1996. Additional sources of resistance to tan spot of wheat. Crop Science 36: 771-777.

Rozen, S., and Skaletsky, H. 2000. Primer3 on the WWW for the general users and for biologist programmers. Methods in Molecular Biology 132: 365-386.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, N.Y.

Sarma, G.N., Manning, V.A., Ciuffetti, L.M., and Karplus, P.A. 2005. Structure of Ptr ToxA: An RGD-containing host-selective toxin from *Pyrenophora tritici-repentis*. The Plant Cell 17:3190-3202.

Schagger, H., and Von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166: 368-379.

Scheffer, R.P., and Briggs, S.P. 1981. Introduction: A perspective of toxin studies in plant pathology. Pages 1-20 in: Toxins in Plant Disease. R.D. Durbin, ed. Academic Press, New York, USA.

Scheffer, R.P., and Livingston, R.S. 1984. Host selective toxins and their role in plant diseases. Science 223: 17-21.

Schilder, A.M.C., and Bergstrom, G.C. 1992. The dispersal of conidia and ascospores of *Pyrenophora tritici-repentis.* Pages 96-99 in: Proceedings from the Second International Tan Spot workshop. L.J. Francl, J.M., Krupinsky, M.P. McMullen, eds. North Dakota Extension Service, ND, USA.

Schilder, A.M.C., and Bergstrom, G.C. 1995. Seed transmission of *Pyrenophora triticirepentis,* causal fungus of tan spot of wheat. European Journal of Plant Pathology 101:81-91.

Singh, P. K., Anderson, J. A., Glover, K. D., Berzonsky, W. A., Elias, E. M., Mergoum, M., Ali, S., and Adhikari, T. B. 2006a. Evaluation of Elite Wheat Germ Plasm for Resistance to Tan Spot. Plant Disease 90: 1320-1325.

Singh, P.K., Mergoum, M, Ali, S., Adhikari, T.B., Elias, E.M., and Hughes, G.R. 2006b. Identification of new sources of resistance to tan spot, Stagonospora nodorum blotch, and Septoria tritici blotch of wheat. Crop Science 46: 2047-2053.

Singh, P.K., and Hughes, G.R. 2006. Inheritance of resistance to the chlorosis component of tan spot of wheat caused by *Pyrenophora tritici-repentis*, races 1 and 3. Euphytica 152:413–420.

Singh, P.K., Mergoum, M., Adhikari, T.B., Shah, T., Ghavami, F., and Kianian, S.F. 2010. Genetic and molecular analysis of wheat tan spot resistance effective against *Pyrenophora tritici-repentis* races 2 and 5. Molecular Breeding 25:369–379.

Singh, P.K., Mergoum, M., Ali, S., Adhikari, T.B., and Hughes, G.R. 2008. Genetic analysis of resistance to *Pyrenophora tritici-repentis* races 1 and 5 in tetraploid and hexaploid wheat. Phytopathology 98:702–708.

Singh, P.K., Singh, R.P., Duveiller, E., Mergoum, M., Adhikari, T.B., and Elias, E.M. 2010. Genetics of wheat-*Pyrenophora tritici-repentis* interactions. Euphytica 172: 1-13.

Stock, W.S., Penner, G.A., and Brule-Babel, A. 1996. A gene for resistance to a necrosis-inducing isolate of *Pyrenophora tritici-repentis* located on 5BL of *Triticum aestivum* cv. Chinese spring. Genome 39: 598-604.

Strelkov, S. E., and Lamari, L. 2003. Host-parasite interactions in tan spot (*Pyrenophora tritici-repentis*) of wheat. Canadian Journal of Plant Pathology 25: 339-349.

Strelkov, S.E. 1998. Purification and mode of action of Ptr *(Pyrenophoratritici-repentis)* chlorosis toxin. M.Sc. thesis, University of Manitoba, Winnipeg, Canada.

Strelkov, S.E. 2002. Occurrence and expression of *ToxB* and comparative virulence of chlorosis-inducing races *of Pyrenophora tritici-repentis*. Ph.D. thesis, University of Manitoba, Winnipeg, Canada.

Strelkov, S.E., Kowatsch, R.F., Ballance, G.M., and Lamari, L. 2006. Characterization of the *ToxB* gene from North African and Canadian isolates of *Pyrenophoratritici-repentis.* Physiological and Molecular Plant Pathology 67: 164-170.

Strelkov, S.E., Lamari, L., and Ballance, G.M. 1998. Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophoratritici-repentis.* Canadian Journal of Plant Pathology 20: 428-435.

Strelkov, S.E., Lamari, L., and Ballance, G.M. 1999. Characterization of a host-specific protein toxin (Ptr ToxB) from *Pyrenophora tritici-repentis.* Molecular Plant-Microbe Interactions 12: 728-732.

Strelkov, S.E., Lamari, L., Sayoud, R., and Smith, R.B. 2002. Comparative virulence of chlorosis-inducing races of *Pyrenophora tritici-repentis.* Canadian Journal of Plant Pathology 24: 29-35.

Summerell, B.A., and Burgess, L.W. 1989. Factors influencing survival of *Pyrenophora tritici-repentis:* water potential and temperature. Mycological Research 93: 41-45.

Sutton, J.C., and Vyn, T.J. 1990. Crop sequences and tillage practices in relation to disease of winter wheat in Ontario. Canadian Journal of Plant Pathology 12: 358-368.

Tomas, A., and Bockus, W.W. 1987. Cultivar-specific toxicity of culture filtrates of *Pyrenophora tritici-repentis*. Phytopathology 77:1337-1340.

Tuori, R.P., Wolpert, T.J., and Ciuffetti, L.M. 1995. Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis.* Molecular Plant-Microbe Interactions 8: 41–48.

Tuori, R.P., Wolpert, T.J., and Ciuffetti, L.M. 2000. Heterologous expression of functional Ptr ToxA. Molecular Plant-Microbe Interactions 14: 456-464.

Valder, P.G., and Shaw, D.E. 1953. Yellow spot disease of wheat in Australia. Proceedings of the Linnean society of N.S.W. 77: 323-330.

Wegulo, S.N., Klein, R.N., and Harveson, R.M. 2006. Tan disease of wheat. http://ianrpubs.unl.edu/epublic/live/g429/build/g429.pdf.

Wilson, R.E. and Loughman, R. 1998. Status of breeding for resistance to *Pyrenophora tritici-repentis* in Western Australia. Canadian Journal of Plant Pathology 20: 419-420.

Wright, K.H. and Sutton, J.C. 1990. Inoculum of *Pyrenophora tritici-repentis* in relation to epidemics of tan spot of winter wheat in Ontario. Canadian Journal of Plant Pathology 12:149-157.

Xu, S. S., Mujeeb-Kazi, A., and Friesen, T. L. 2004. Seedling resistance to tan spot and Stagonospora nodorum blotch in synthetic hexaploid wheats. Crop Science 44: 2238-2245.

Yoder, O. C. 1980. Toxins in pathogenesis. Annual Review of Phytopathology 18: 103-129.

Zillinsky, F. 1983. Common diseases of small grain cereals, a guide to identification. Mexico, DF, CIMMYT. 141 pp.