Occurrence and Expression of *ToxB* and Comparative Virulence of Chlorosis-Inducing Races of *Pyrenophora tritici-repentis*

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ACKNOWLEDGMENTS

I would like to thank:

My advisor, Dr. L. Lamari, for his guidance and support during my studies.

The members of my committee, Drs. Ballance, Vessey, Huebner and Ciuffetti, for reading my thesis and for all of their suggestions.

The staff of the Plant Science Department, including R. Kowatsch, R. Smith, D. Durnin, B. Luit, the office staff (particularly M. Blouw), and the greenhouse staff.

My fellow graduate students, past and present, for their friendship.

My family, for their constant support.

My wife Ileana, for always being there for me.

The University of Manitoba and the Canadian Wheat Board for financial assistance.

FOREWORD

This thesis is written in manuscript style. A general introduction and review of the literature precedes the four manuscripts that comprise the main part of the thesis. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion. The first manuscript (Section 3.0) has been published in the Canadian Journal of Plant Pathology (24:1-7). The second one (Section 4.0) will be submitted to Phytopathology. A general discussion and a list of literature cited follow the manuscripts.

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ABSTRACT

Tan spot is an important foliar disease of wheat caused by the fungus *Pyrenophora tritici-repentis*, some isolates of which produce the host-specific, chlorosis-inducing toxin Ptr ToxB. The chlorosis symptoms produced by various isolates of *P. tritici-repentis* were compared, and the occurrence of the *ToxB* gene, which codes for Ptr ToxB, was investigated among isolates representing all known races of the fungus. Isolate Alg-H2, collected in Eastern Algeria, has a virulence pattern that combines those of races 3 and 5, and was therefore classified in a new race, designated race 6. Isolate 92-171R5, collected on the Manitoba-Saskatchewan border, belongs to race 5, but is much less aggressive than other race 5 isolates. Ptr ToxB was purified to homogeneity from Alg-H2 culture filtrate, and the presence of a toxic principle, with the same specificity as Ptr ToxB, was also demonstrated in 92-171R5 culture filtrate. A form of the *ToxB* gene was found to occur in races 3, 4, 5 and 6, but was absent in races 1 and 2. Northern blotting analysis revealed the presence of Ptr ToxB mRNA in total RNA extracted from races 5 and 6 isolates Alg 3-24 and Alg-H2. Using RT-PCR, the presence of Ptr ToxB mRNA was also confirmed in total RNA from ungerminated spores of isolates D308 (race 3), 90-2 (race 4) and 92-171R5. PCR-products were cloned and sequenced, and the cDNA sequences coding for mature toxin were found to be 100% homologous among all isolates except 90-2, which exhibited only 86% homology with the others. Ptr ToxB appeared to have no direct effect on plant water or CO_2 exchange with the atmosphere. However, in sensitive tissue it caused significant declines in the relative pool sizes of foliar Ser, Gly and Ala, while inducing increases in Glu + Gln, Pro and the branched chain amino acids.

1.0 INTRODUCTION

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoem.), is an important foliar disease of wheat worldwide. While its occurrence as an endemic disease of wheat has been known in many regions since the 1940s (Rees et al. 1982), there has been an increased incidence of this disease in recent years, as a consequence of the shift towards stubble retention by farmers (Rees 1982; Bockus 1998). This in turn has elevated the economic significance of tan spot, as severe epidemics may result in yield losses of up to 50% (Rees et al. 1982). Tan spot consists of two distinct symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier 1989a). Host reaction to the pathogen can be described in a qualitative manner (Lamari and Bernier 1989a), as can variation for virulence in the pathogen (Lamari and Bernier 1989b). Thus, isolates of *P. tritici-repentis* were originally grouped into four pathotypes based on their ability to cause necrosis and/or chlorosis on differential wheat cultivars (Lamari and Bernier 1989b). This symptom-based classification system allowed for the description of a maximum of four pathotypes: pathotype 1 (P1) causes both necrosis and chlorosis (nec+chl+), pathotype 2 (P2) causes only necrosis (nec+chl-), pathotype 3 (P3) causes only chlorosis (nec-chl+), and pathotype 4 (P4) is avirulent and produces neither symptom (nec-chl-).

The identification of Algerian fungal isolates possessing a virulence pattern different from those reported in North America served to illustrate the limitations of the pathotype system in describing isolates of *P. tritici-repentis* (Lamari et al. 1995). Various isolates collected in Algeria belong to pathotype 3, in that they can induce chlorosis but not necrosis on hexaploid wheat differentials (Lamari et al. 1995).

However, while North American pathotype 3 isolates are virulent on wheat line 6B365 and avirulent on cv. Katepwa, these Algerian isolates are virulent on cv. Katepwa and avirulent on line 6B365 (Lamari et al. 1995). Hence, a race designation was proposed to classify isolates of *P. tritici-repentis* based on their virulence on differential wheat lines (Lamari et al. 1995). This race-based system was a refinement of the symptom-based classification system, since only the size and effectiveness of the differential set limit the number of races that can be described. Under the race classification system, the isolates within the original pathotypes 1 to 4 have been reclassified as belonging to races 1 to 4, respectively, while the Algerian chlorosis-inducing isolates have been classified as members of race 5 (Lamari et al. 1995). New races of *P. tritici-repentis* will be identified as isolates are discovered with different virulence patterns.

A host-specific toxin termed Ptr ToxA, produced by races 1 and 2 isolates of *P. tritici-repentis*, was shown to be responsible for the development of the necrotic symptom in susceptible wheat lines and cultivars (Lamari and Bernier 1989c). This toxin has been purified to homogeneity from the culture filtrates of necrosis-inducing isolates, and characterized by several independent research groups (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997). Ptr ToxA is a 13.2 kDa protein, and genomic and cDNA clones of *ToxA*, the gene coding for the toxin, have been obtained (Ballance et al. 1996; Ciuffetti et al. 1997). Ptr ToxA shows the same host-specificity as the fungal isolates from which it is obtained, and has been recognized as an important factor in the development of tan spot (Tomás and Bockus 1987; Ballance et al. 1989; Lamari and Bernier 1991).

A second host-specific toxin, capable of inducing chlorosis in various wheat lines and cultivars, was identified in race 5 culture filtrates of *P. tritici-repentis* (Orolaza et al. 1995). This toxin, designated Ptr ToxB, has been purified and characterized (Strelkov et al. 1999). Like Ptr ToxA, it is a small protein, 6.61 kDa in size (Strelkov et al. 1999), and its coding region has been recently cloned (Martinez et al. 2001; G.M. Ballance, *unpublished data*). Also like Ptr ToxA, Ptr ToxB possesses a host specificity identical to that of the isolates from which it has been obtained (Orolaza et al. 1995). This and other evidence strongly suggest that Ptr ToxB is responsible for the development of chlorosis in susceptible hosts (Orolaza et al. 1995). The induction of chlorosis in response to the toxin appears to result from the active oxygen-mediated photo-oxidation of chlorophyll, likely as a consequence of an inhibition of photosynthesis (Strelkov et al. 1998). However, the exact nature of the effect(s) of Ptr ToxB on sensitive tissue remains to be elucidated.

Different research groups previously referred to Ptr ToxA as Ptr necrosis toxin, Ptr toxin or ToxA, while Ptr ToxB was formerly referred to as Ptr chlorosis toxin. In an attempt to standardize toxin nomenclature in the tan spot system, the toxins were renamed Ptr ToxA and Ptr ToxB (Ciuffetti et al. 1998). Chlorosis in response to infection by races 1 and 3 isolates appears to be due to their production of a third host-specific toxin, termed Ptr ToxC, which has been partially purified (Effertz et al. 2002).

The principal objectives of this research were: (1) to examine the occurrence and expression of the *ToxB* gene among isolates of *P. tritici-repentis* representing different races, (2) to obtain cDNA clones of Ptr ToxB mRNA from those isolates in which it is present, (3) to use immunological techniques to detect the Ptr ToxB protein in the

isolates, (4) to compare the virulence pattern and aggressiveness of the various chlorosis-inducing isolates, and characterize new races found among isolates obtained from several locations, and (5) to further characterize the effect of Ptr ToxB on sensitive and insensitive wheat tissue.

2.0 LITERATURE REVIEW

2.1 Tan Spot of Wheat

Tan spot of wheat is caused by the fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis*), a homothallic ascomycete. This disease, also known as yellow spot, generally begins as tan-brown flecks on the leaves of affected plants. These flecks expand into brown coloured, oval shaped spots surrounded by a yellow margin. As the disease progresses, these spots can coalesce, producing large areas of dead and chlorotic tissue. Tan spot has been identified throughout the major wheat growing regions of the world, including Canada, Australia, and the United States (Hosford 1982; Wiese 1987). While its occurrence as an endemic disease of wheat has been known since the 1940s (Rees et al., 1982), it is only in recent decades that tan spot has become an important constraint to wheat production (Rees and Platz 1992). Yield losses can be very high; Rees et al. (1982) reported losses of up to 50% in severe epidemics. However, losses of 5 to 10% are not uncommon (Hosford 1982), particularly since most wheat cultivars are susceptible to tan spot (Martens et al. 1988).

The increased incidence of tan spot in recent years can be largely attributed to the widespread adoption of conservation tillage techniques by farmers. Tan spot is caused by a stubble-borne pathogen and hence, reduced tillage practices allow for longer survival of the pathogen on crop residues on the soil surface, leading to an increase in inoculum levels. Changes in cultivar genotypes may have also played a role in the increased importance of tan spot; many of the semi-dwarf wheat varieties introduced after 1960 have a high susceptibility to this disease (Rees and Platz 1992). Compounding the problem is the fact that *P. tritici-repentis* has the widest host range of any member of the

genus *Pyrenophora* (Shoemaker 1962). *P. tritici-repentis* has been identified on many species of grasses, as well as on several cereal species, such as triticale (*Triticum secale*) and rye (*Secale cereale*) (Shoemaker 1962; Morral and Howard 1975; Krupinsky 1992). This large host range allows *P. tritici-repentis* to overwinter on a large number of grasses, which could provide inoculum to initiate tan spot epidemics in successive wheat crops (Krupinsky 1986).

The tan spot syndrome is associated with two distinct symptoms, tan necrosis and/or chlorosis. The development of each symptom is the result of specific interactions between individual isolates of *P. tritici-repentis* and wheat genotypes (Lamari and Bernier 1989a). Some wheat cultivars develop both symptoms when challenged with appropriate isolates of the fungus, while others selectively develop necrosis or chlorosis (Lamari and Bernier 1989a; Lamari et al. 1991). Isolates of *P. tritici-repentis* were originally grouped into four pathotypes based on their ability to induce necrosis and chlorosis (pathotype 1), necrosis only (pathotype 2), chlorosis only (pathotype 3), or neither symptom (pathotype 4) in differential wheat genotypes (Lamari and Bernier 1989b). The identification of Algerian pathotype 3 isolates that can induce chlorosis in wheat genotypes previously known to be resistant to all isolates of this pathotype led to the adoption of a race classification system (Lamari et al. 1995). In this system, modeled on that of the rusts, isolates of *P. tritici-repentis* are classified into races based on their virulence on a wheat differential set. Only the size and effectiveness of the differential set limit the number of races that can be described. This is in contrast with the pathotype classification system, which can describe a maximum of only four pathotypes (Lamari et al. 1995). At the beginning of the present Ph.D. program, five races had been identified.

Races 1 to 4 correspond to the original pathotypes 1 to 4, respectively, and race 5 includes the Algerian pathotype 3 isolates. The five races and their host ranges are summarized in Table 2.1.

2.2 Toxin Involvement in Tan Spot

2.2.1 Toxins from *Pyrenophora tritici-repentis*

The chlorosis and necrosis symptoms associated with tan spot result from the production, by *P. tritici-repentis*, of multiple host-specific toxins. To date, three host-specific toxins have been identified from the culture filtrates and/or spore germination fluids of various isolates of the fungus. Necrosis in hexaploid wheat lines or cultivars is due to the production, by races 1 and 2 isolates, of Ptr ToxA. The mature form of this toxin is a 13.2 kDa protein, coded for by a gene termed *ToxA*, which has been cloned by two independent research groups (Ballance et al. 1996; Ciuffetti et al. 1997). Chlorosis in response to infection by race 5 isolates is due to the production of a second host-specific toxin, designated Ptr ToxB. This toxin is also a protein, with a mass of 6.61 kDa (Strelkov et al. 1999). Genomic and cDNA clones of *ToxB*, the gene that codes for Ptr ToxB, have been recently obtained (Martinez et al. 2001; G.M. Ballance, *unpublished data*). Several lines of evidence, including the fact that both Ptr ToxA and Ptr ToxB possess host specificities identical to that of the isolates from which they were obtained, suggest that these toxins are pathogenicity factors (Lamari and Bernier 1991; Orolaza et al. 1995). The third host-specific toxin known to be produced by *P. tritici-repentis* is Ptr ToxC. It is synthesized by races 1 and 3 isolates of the fungus, and appears to cause chlorosis in wheat genotypes susceptible to those races (Effertz et al. 2002). Ptr ToxC is

Table 2.1. Reaction of eight wheat genotypes to five races of *Pyrenophora tritici-repentis* differing in ability to cause tan necrosis and chlorosis.

	Disease Rating[a]				
Cultivar or line[b]	Race 1	Race 2	Race 3	Race 4	Race5
Glenlea	4-5N	4-5N	1-2	1	1-2
Katepwa	5N	5N	1-2	1	4-5C
6B662	1-2	1-2	1-2	1	4-5C
6B365	5C	1-2	5C	1	1-2
Erik	1	1	1	1	1
Salamouni	1	1	1	1	1
4B1149	1	1	1	1	1
Coulter	4N	4N	4-5N	1	4-5N

[a]Plants were rated on a scale of 1-5 based on lesion type, where 1 and 2 represent resistance and 3-5 susceptibility. N = tan necrosis and C = chlorosis.

[b]All cultivars and lines listed are hexaploid except for line 4B1149 and cv. Coulter, which are tetraploid.

the least characterized of the three toxins. It has only been partially purified, and seems to be a polar, nonionic, small molecular weight molecule (Effertz et al. 2002). There is evidence for the existence of other host-specific toxins in the tan spot system (L. Lamari, *personal communication*; Tuori et al. 1995), but these are yet to be identified.

2.2.2 Mode of Action Studies

Ptr ToxA. Little is known regarding the exact nature of the molecular mechanisms that result in the necrosis caused by Ptr ToxA. Development of necrosis in response to this toxin has been shown to be a temperature-dependent process (Lamari and Bernier 1994; Kwon et al. 1996; Kwon et al. 1998). At temperatures above 27^0C, necrosis (Lamari and Bernier 1994) and electrolyte leakage (Kwon et al. 1996) fail to develop in sensitive wheat leaves treated with the toxin. High temperatures have been postulated to interfere with the binding of Ptr ToxA and its putative receptor (Lamari and Bernier 1994), or to interrupt the signaling pathway downstream from toxin recognition (Kwon et al. 1996). However, no receptor has as of yet been demonstrated, although two groups are using the yeast two-hybrid system to identify interactions between Ptr ToxA and proteins from toxin-sensitive wheat (Ciuffetti and Tuori 1999; G.M. Ballance, *personal communication*).

Deshpande (1993), using cell suspensions and calli, found no negative effect of Ptr ToxA in cultures derived from toxin-sensitive vs. insensitive tissue. However, cells in callus cultures and cell suspensions are undifferentiated and may therefore be insensitive to the toxin (Deshpande 1993). Furthermore, Ptr ToxA may not have been taken up from the media in sufficient quantities to produce an effect (Deshpande 1993). Kwon et al. (1996) developed an electrolyte leakage assay to quantify the reaction of wheat leaves to

Ptr ToxA. They observed that toxin exposure for 4 h or more enhanced electrolyte leakage from a toxin-sensitive wheat line, but not from a toxin-insensitive cultivar (Kwon et al. 1996). The toxin exposure times required for leakage to develop led Kwon et al. (1996) to conclude that the effect of Ptr ToxA on the plasmalemma is secondary and occurs relatively late in the development of necrosis. Toupin (2000), using microscopic techniques, found that in the early stages of toxin-induced disruption, certain cells appear to have a damaged tonoplast. Based on this observation, he suggested that the tonoplast of toxin-sensitive cells might be a primary target of toxin activity (Toupin 2000).

Another study investigated the involvement of wheat metabolism in the action of Ptr ToxA (Kwon et al. 1998). Kwon et al. (1998) found that necrosis and electrolyte leakage in response to Ptr ToxA-treatment did not develop when leaves were incubated at 4^0C, but that this inhibition of toxin action by cold was reversible. In addition, toxin-induced electrolyte leakage was reduced by the co-application of sodium vanadate, an inhibitor of H^+-ATPases (Kwon et al. 1998). However, the protection vanadate afforded decreased as time increased, and ultimately it did not prevent development of necrosis. Inhibitors of transcription (α-amanitin and cordycepin) and translation (cycloheximide) reduced the level of toxin-induced electrolyte leakage as well, while cycloheximide also prevented development of the necrosis symptom (Kwon et al. 1998). From their observations, Kwon et al. (1998) concluded that action by Ptr ToxA requires active host-processes, including transcription, translation, and functional H^+-ATPases.

Ptr ToxB. Preliminary studies into the mode of action of Ptr ToxB revealed that the development of chlorosis in response to this toxin is light dependent; chlorosis does not develop in darkness, and is much weaker under reduced light conditions (N.P. Orolaza

and L. Lamari, *personal communication*). Furthermore, development of the chlorosis symptom was observed to be independent of tissue age. These results suggested that toxin-induced chlorosis is a consequence of photochemical bleaching, and led Strelkov et al. (1998) to investigate the physiological development of chlorosis in more detail.

Using partially purified Ptr ToxB, Strelkov et al. (1998) found that the toxin did not interfere with the accumulation of chlorophyll in etiolated seedlings exposed to light. This finding, consistent with the earlier observation that toxin-induced chlorosis is independent of tissue age, indicated that chlorosis results from chlorophyll degradation rather than inhibition of chlorophyll synthesis (Strelkov et al. 1998). Moreover, Strelkov et al. (1998) corroborated the prior conclusion that development of chlorosis in response to Ptr ToxB is strictly light dependent. This result conformed to the hypothesis that the degradation of chlorophyll is due to photochemical bleaching (Strelkov et al. 1998). Further evidence for photochemical bleaching was obtained in studies with active oxygen (AO) scavengers (Strelkov et al. 1998). The compound p-benzoquinone, an AO scavenger that quenches triplet chlorophyll and singlet oxygen, prevented development of the chlorosis symptom (Strelkov et al. 1998). High performance liquid chromatography chlorophyll degradation profiles were also consistent with photo-oxidation (Strelkov et al. 1998).

Photo-oxidation can result from an inhibition of photosynthesis or from the failure of the normal photoprotective mechanisms of plants. Since carotenoids are important in the dissipation of the excess energy of chlorophyll molecules and in the detoxification of AO species, Strelkov et al. (1998) examined the possibility that toxin-induced chlorosis is due to a carotenoid deficiency. They found that carotenoid levels declined in toxin-

treated sensitive tissue, but that this decline was concurrent with and smaller than that of the chlorophylls, suggesting it was simply a reflection of widespread AO-mediated damage (Strelkov et al. 1998). However, the effect of Ptr ToxB on the activity of the various antioxidant enzymes involved in photoprotection, such as superoxide dismutase and ascorbate peroxidase, has not been studied. If their function were to be compromised, the ability of plant cells to detoxify AO species would be reduced, leading to extensive damage. Nevertheless, based on their results, Strelkov et al. (1998) hypothesized that Ptr ToxB directly or indirectly inhibits photosynthesis, resulting in chlorophyll degradation, as illuminated thylakoid membranes become incapable of dissipating excitation energy. Most of the studies conducted with partially purified Ptr ToxB have been repeated with pure toxin and the same results obtained (S.E. Strelkov, *unpublished data*).

Ptr ToxC. The mode of action of Ptr ToxC remains a complete mystery; no studies characterizing the development of chlorosis in response to this toxin have been published. Therefore, not even the most basic questions, such as whether the toxin inhibits chlorophyll synthesis or promotes its degradation, have been answered.

2.3 Plant Pathogens and Host-Resistance

2.3.1 General Aspects of Host-Pathogen Specificity

Plants are resistant to most potential pathogens. However, some microorganisms are able to overcome this resistance, known as basic or nonhost resistance, and become parasites of a particular species. To understand how nonhost resistance is related to other more specific types of resistance, such as the resistance of a wheat cultivar to a particular race of rust, we must first consider host-pathogen specificity. Heath (1981a) defined two

types of specificity: (1) plant species specificity, which determines host species range, and (2) cultivar specificity, which determines cultivar range within a particular host species. Others have proposed similar ideas. Day (1976) distinguished between host-pathogen interactions that determine whether or not a plant is a host for a particular pathogen, and those that determine the gene-for-gene specificity between a pathogen and host. Ellingboe (1976) used the term basic compatibility to describe the situation wherein a microorganism is a pathogen of a particular plant species, and suggested that the gene-for-gene relationship is superimposed on this basic compatibility. Heath (1981a) summarized and combined these ideas into a generalized model of host-pathogen specificity.

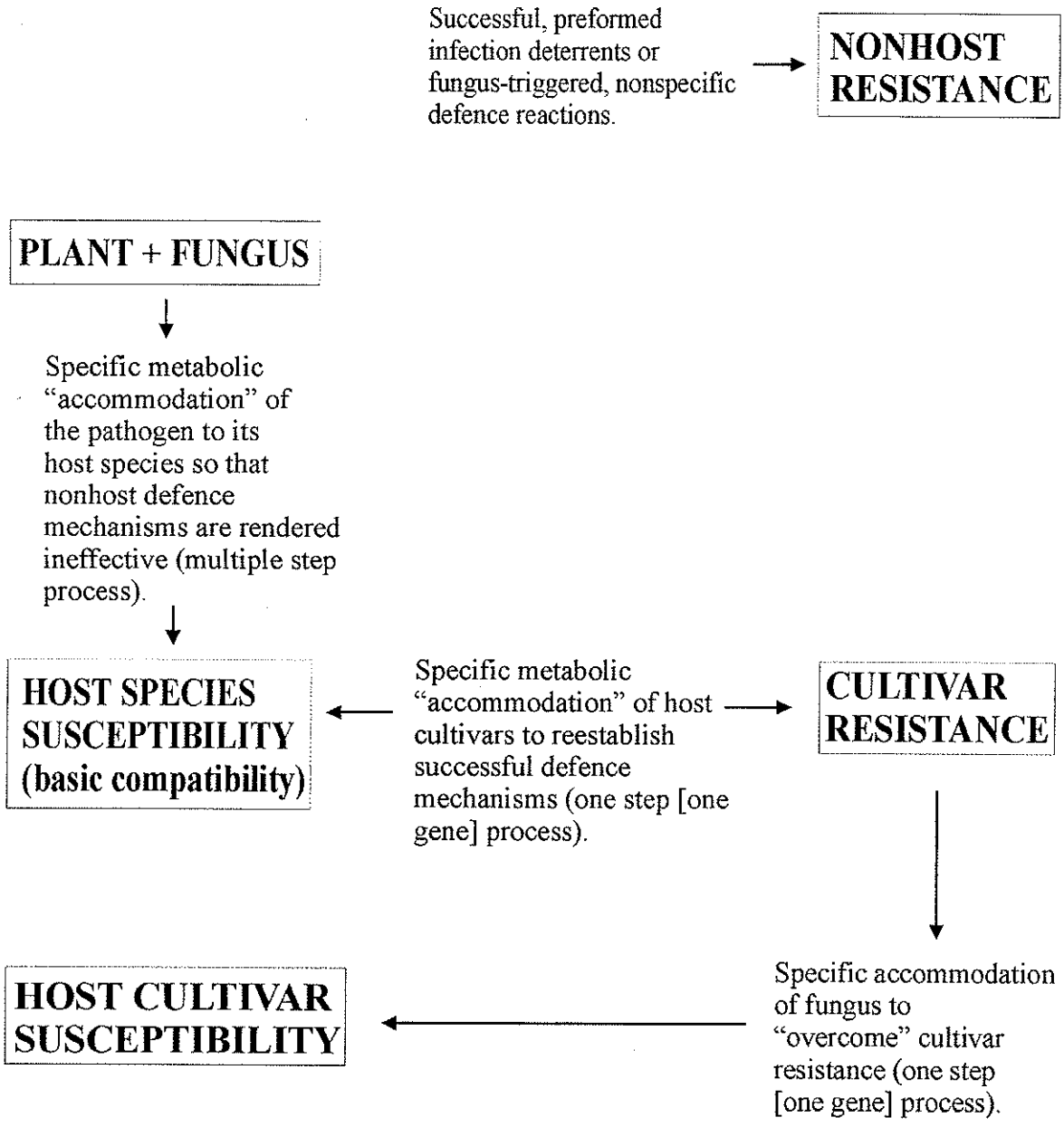
2.3.2 Basic Compatibility Model of Specificity

In her model, Heath (1981a) discussed how microorganisms first overcome nonhost resistance, and how the response of the host in turn results in cultivar resistance. Nonhost resistance is genetically complex, and consists of many different components. There are preformed physical or chemical deterrents to infection, as well as a range of non-specific defence reactions, which are elicited upon attempted infection by a potential pathogen (Heath 1981a). These include processes such as lignification and antimicrobial phytoalexin accumulation, which can be triggered by a wide range of fungal cell wall components (Agrios 1997). Thus, as would be expected given their nonspecific nature, basic resistance mechanisms can be elicited by compounds common to many pathogens (Heath 1981a). To successfully colonize a host, the pathogen must adapt itself to its host so as not to trigger the mechanisms of nonhost resistance (Heath 1981a). The ways in which this can be accomplished, thereby establishing basic compatibility, are numerous

(Ellingboe 1976). For example, the pathogen could develop tolerance to certain antimicrobial compounds, or it could produce an enzyme to make them innocuous (Heath 1981a). Alternatively, it could produce a host-selective toxin, as is the case with some plant pathogens, which would function as a pathogenicity factor (Scheffer 1991).

Once basic compatibility is established between a pathogen and its host species, the host is susceptible to infection by the pathogen. Hence, there is strong selection pressure on the host to develop some other form of resistance to arrest or reduce infection. This type of resistance, superimposed over nonhost resistance, is what Heath (1981a) referred to as cultivar resistance. Thus, to initially establish basic compatibility, it is the pathogen that must adapt. However, to re-establish resistance, in the form of cultivar resistance, it is the host who must adapt (Heath 1981a). It may do this in a variety of ways. For instance, if the pathogen overcame basic resistance by producing a host-specific toxin, then the host may eliminate or alter the receptor site for this toxin, rendering it ineffective (Scheffer 1991). Alternatively, active defence reactions could be triggered by some fungal product that the host could recognize. Obviously, the host would have to develop the ability to recognize this product (Heath 1981a). If this occurred, the host gene controlling fungal recognition would become the resistance gene, while the fungal gene controlling the production of the fungal product would become the avirulence gene. Hence, a gene-for-gene relationship would be established (Heath 1981a). Heath's (1981a) model of host-parasite specificity is summarized in Figure 2.1. It was later termed the basic compatibility model of specificity (Heath 1991).

Figure 2.1. Heath's (1981a) model of host-parasite specificity.


Successful, preformed infection deterrents or fungus-triggered, nonspecific defence reactions.
NONHOST RESISTANCE
PLANT + FUNGUS
Specific metabolic "accommodation" of the pathogen to its host species so that nonhost defence mechanisms are rendered ineffective (multiple step process).
HOST SPECIES SUSCEPTIBILITY (basic compatibility)
Specific metabolic "accommodation" of host cultivars to reestablish successful defence mechanisms (one step [one gene] process).
CULTIVAR RESISTANCE
Specific accommodation of fungus to "overcome" cultivar resistance (one step [one gene] process).
HOST CULTIVAR SUSCEPTIBILITY

2.3.3. Pathogenicity Factors

To establish basic compatibility, the pathogen must evolve certain characteristics or pathogenicity factors (Heath 1987). Heath (1987) loosely defined the term "pathogenicity factor" as a fungal attribute that matches basic resistance mechanisms. She stated that it is likely that many pathogenicity factors are required to induce susceptibility in a host species (Heath 1987). However, Yoder (1980) defined a pathogenicity factor more specifically as a molecule required to cause disease. It should be clarified that Yoder (1980) used the word "pathogenicity" to mean the ability to cause disease, and "virulence" to refer to the degree of pathogenicity or the extent of disease. Others (e.g. Jarosz and Davelos 1995) substitute the term "aggressiveness" in place of virulence, to avoid confusion with the word "virulent," which also means pathogenic (Zadoks and Schein 1979).

Despite differences in the definitions of pathogenicity factors, they can still be regarded as either molecules or attributes necessary to overcome resistance and cause disease. If a saprophyte or non-pathogen is to become parasitic, it must evolve or acquire the necessary pathogenicity factor(s). To better understand which factors are missing among saprophytes, the interactions between nonhost bean plants and a diverse group of fungal saprophytes and parasites were compared (Fernandez and Heath 1986; Heath 1987). It was found that with respect to behaviour on and in dead bean leaves, there was little difference among the saprophytes and nonbiotrophic parasites tested (Fernandez and Heath 1986; Heath 1987). However, while the saprophytes failed to germinate or germinated poorly on intact leaf surfaces, the pathogens showed a high degree of germination. Furthermore, all the pathogens occasionally managed to penetrate the leaf

via stomata, and some even attempted to penetrate directly (Fernandez and Heath 1986; Heath 1987). Based on these observations, Heath (1987) suggested that the saprophytes lacked at least two important pathogenicity factors possessed by the pathogens: (1) the ability to germinate in low nutrient environments such as leaf surfaces, and (2) the ability to penetrate tissue in the absence of nutrient gradients established by leakage from inside the leaves. Also examined were the various responses of the bean leaves, such as fluorescence and callose deposition, to treatment with saprophyte and pathogen spores (Fernandez and Heath 1986; Heath 1987). The pathogens elicited most of these responses with less frequency and strength than did the saprophytes. According to Heath (1987), the capacity not to trigger a particular response can also be regarded as a pathogenicity factor. The bean rust fungus, *Uromyces appendiculatus*, induces none of the mechanisms for basic resistance (Heath 1981b). Thus, it possesses all the necessary pathogenicity factors to suppress or avoid triggering these reactions (Heath 1987).

There is also strong evidence that pathogenicity factors as defined by Yoder (1980) can be very important in overcoming basic or cultivar resistance. The acquisition of a single gene for host-specific toxin production can convert a benign, opportunistic pathogen into a virulent, specialized pathogen capable of causing severe epidemics (Scheffer 1991). A classic example of this is the appearance of Southern leaf blight of corn, caused by *Cochliobolus heterostrophus* (teleomorph of *Helminthosporium maydis*) (Scheffer and Livingston 1984; Graniti 1991; Scheffer 1991). Prior to 1968, *C. heterostrophus* was confined to the southeastern edge of the U.S. corn belt, where it caused a minor leaf disease in maize. However, a new race of *C. heterostrophus*, termed race T, appeared in 1968. It developed from the old race of the fungus, race O, and was

identical to it except that it possessed the ability to produce a host-selective toxin, HMT toxin. Race T caused devastating epidemics of the maize crop in North America in 1970 and 1971 (Scheffer and Livingston 1984; Graniti 1991; Scheffer 1991). The epidemics were possible because of the widespread use of Texas male sterile (Tms) maize lines; HMT toxin uncouples respiration in mitochondria from plants with Tms cytoplasm, but has no effect on mitochondria from resistant maize (Bednarski et al. 1977). Thus, the basis for selectivity and virulence by *C. heterostrophus* race T was the production of HMT toxin. When Tms cytoplasm maize was abandoned, race T soon became rare. The acquisition of pathogenicity factors such as host-selective toxins not only aids in the adaptation of pathogens to selected hosts, but can also provide the ecological and genetic isolation necessary for evolutionary change and speciation (Scheffer 1991).

2.4 Phytotoxins

2.4.1 Host-Specific vs. Host-Nonspecific Toxins

Many bacterial and fungal plant pathogens produce compounds that are toxic to plants; these substances are generally referred to as phytotoxins (Strobel 1982; Graniti 1991). As a group, these compounds vary dramatically in both size and structure, although most known phytotoxins are low molecular weight secondary metabolites (Strobel 1982; Scheffer and Livingston 1984; Graniti 1991). Phytotoxins are either host-specific (host-selective) or host-nonspecific (host-nonselective). Nonspecific phytotoxins do not reproduce the patterns of resistance and susceptibility of the host to the pathogen (Graniti 1991). Plants of a genotype that are resistant to a particular toxin-producing pathogen may be sensitive to the isolated toxin. Therefore, while some nonspecific toxins contribute to symptom development in the diseases in which they occur, they are

not primary determinants of host range (Ballio and Graniti 1991; Walton 1996). Host-specific toxins, on the other hand, affect only plants of a genotype susceptible to the pathogen (Graniti 1991) and act as virulence or pathogenicity factors (Walton 1996). Furthermore, they are able to produce, in susceptible hosts, the symptoms of the natural infection at low or physiological concentrations (Goodman et al. 1986; Graniti 1991). All known bacterial phytotoxins as well as a majority of the fungal ones are host-nonspecific (Graniti 1991; Walton 1996). However, given that all toxins identified thus far from *P. tritici-repentis* are host-selective, the focus here will be on host-specific toxins.

2.4.2 Characteristics of Host-Specific Toxins

As is the case with phytotoxins in general, the vast majority of host-specific toxins are low molecular weight secondary metabolites with diverse structures (Walton and Panaccione 1993; Walton 1996). Until recently, the only known exceptions came from the tan spot system; both Ptr ToxA and Ptr ToxB are ribosomally synthesized polypeptides (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997; Strelkov et al. 1999). However, another proteinaceous host-specific toxin, produced by *Alternaria brassicicola*, has now been reported (Otani et al. 1998). Like many other secondary metabolites, most host-specific toxins belong to families of compounds, each member of which is produced in a different amount and has a different potency (Scheffer and Livingston 1984). Chemical classes that are represented in the host-specific toxins include polyketides, saccharides, terpenoids, cyclic peptides and compounds of uncertain biogenesis (Walton 1996).

Typical bioassays for both host-specific and nonspecific toxins include development of chlorosis or necrosis upon infiltration into sensitive tissue, inhibition of root growth, and stimulation of electrolyte leakage (Walton 1996). Electrolyte leakage is most likely a consequence of the inability of dying cells to maintain membrane integrity (Durbin 1991). Nonetheless, it has also been hypothesized to be a critical biological effect of toxins because it would allow low molecular weight nutrients to diffuse out of cells and into the apoplast, where they could be easily absorbed by the pathogen (Ballio and Graniti 1991; Durbin 1991). Many of the fungi known to produce host-specific toxins are leaf pathogens, and since low molecular weight toxins are relatively mobile, initial evidence for the involvement of a host-specific toxin in a particular disease has often come from occurrence of symptoms away from the site of infection.

Toxins generally kill cells, and host-specific toxins are active at concentrations within a range of approximately 10 pM to 1 μM (Walton 1996). Their degree of host-selectivity or specificity ranges from 100 to $>10^6$ fold (Walton 1996). Nevertheless, not all toxins are toxic under all conditions. For instance, light inhibits the symptoms of AM-toxin toxicity without altering the response of the host plant to the pathogen (Tabira et al. 1989). HC-toxin does not kill nondividing leaf mesophyll protoplasts but actually promotes their survival (Wolf and Earle 1991). There is also strong evidence to suggest that the toxicity of host-specific toxins requires active participation (transcription and translation) on the part of the host cell. Inhibitors of protein and RNA synthesis protect sensitive cells against some host-specific toxins. For example, it was found that cycloheximide (an inhibitor of translation), and aminitin and cordycipin (inhibitors of transcription) reduced toxin-induced electrolyte leakage by as much as 90 % in response

to Ptr ToxA (Kwon et al. 1998). Inhibitors of transcription and translation have also been found to protect sensitive cells against various other host-specific toxins, including AM-toxin, AK-toxin, AAL-toxin, victorin and peritoxin (Walton and Panaccione 1993; Walton 1996).

2.4.3 Basis for Specificity of Host-Specific Toxins

The high specificity of host-selective toxins has been postulated to be a result of the presence, in sensitive cells, of receptors for these toxins - a model first proposed by Pringle and Scheffer (1964). It is hypothesized that the presence of the appropriate receptor is required for action by a particular toxin, and since host-specific toxins are pathogenicity factors, in the absence of the receptor the plant is resistant. This model is highly compatible with the dominant sensitivity that has been observed in response to many host-specific toxins, for instance victorin and Ptr ToxA. However, Walton and Panaccione (1993) distinguish between the concepts of receptors in plant pathology and that of the better-characterized receptors in mammals. They point out that in mammalian systems, receptors are involved in the internal homeostasis of an organism, are linked to signal transduction pathways, and are frequently membrane-localized; the concept of receptors is broader in plant pathology (Walton and Panaccione 1993). In plant-mircrobe interactions, an enzyme that is the site of action of a biologically active compound can be considered its receptor, even if the binding is accidental. For example, the site of action of tentoxin is the CF_1 domain of chloroplast ATPase, and thus this enzyme can be considered its receptor (Steele et al. 1976), even though it is not a receptor in the classical sense.

2.4.4 Toxin and Gene-for-Gene Models of Host-Pathogen Interaction

In the situation described above, a pathogen-produced molecule is recognized by a host-produced receptor, resulting in the development of disease or compatibility. This is known as the toxin model of host-pathogen interaction (Loegering 1978), in which the compatible interaction is the basis for specificity. The toxin model contrasts with, but does not necessarily contradict, the gene-for-gene model of host-pathogen interaction (Flor 1971). In the gene-for-gene model, a pathogen produces an avirulence gene product or elicitor, which is recognized by a resistance gene-coded receptor molecule on the host cell (Agrios 1997). This recognition or binding event by the host receptor triggers a defense response in the plant, leading to incompatibility and no disease development (Agrios 1997). Therefore, in the gene-for-gene model, the incompatible interaction is the basis for specificity.

Interestingly, there is one known case in which the receptor that confers resistance to one disease appears to be the same one that confers susceptibility to another. The *Pc-2* gene, discovered in Uruguay, gives good resistance to *Puccinia coronata*, causal agent of crown rust of oats. In the 1930s, crown rust was an important disease, and in breeding for resistance against it, the *Pc-2* gene was introgressed into the major oat varieties in the United States (Walton 1996). This resulted in major epidemics of a previously unknown disease, Victoria blight of oats, caused by the fungus *Cochliobolus victoriae*. This pathogen produces the host-specific toxin victorin (Mehean and Murphy 1947), which is a pathogenicity factor that allows it to specifically infect oat varieties carrying the *Pc-2* gene (Wolpert et al. 1994). The gene that confers susceptibility to *C. victoriae*, termed *Hv-1* or *Vb*, is either the same gene as, or is tightly linked to, *Pc-2* (Rines et al. 1985;

Mayama et al. 1995). Thus, the *Pc-2* gene presumably codes for a receptor that binds an elicitor from *P. coronata*, triggering a defense response against this pathogen. However, this appears to be the same receptor that also binds victorin, resulting in susceptibility to *C. victoriae*.

3.0 COMPARATIVE VIRULENCE OF CHLOROSIS-INDUCING RACES OF *PYRENOPHORA TRITICI-REPENTIS*

3.1 Abstract

The fungal pathogen *Pyrenophora tritici-repentis* causes tan spot, an important foliar disease of wheat worldwide. To date, five races of this pathogen have been described. We report the existence of a sixth race, and compare the chlorosis symptoms produced by various isolates of *P. tritici-repentis* representing different races. Isolate Alg-H2, collected in eastern Algeria, has a virulence pattern that combined those of races 3 and 5. Thus, we propose that this isolate be classified in a new race, designated race 6. Isolate 92-171R5, collected on the Manitoba-Saskatchewan border, possesses the same virulence pattern as race 5 isolates. However, it was much less aggressive than other race 5 isolates, based on the size of the lesions and the extent of the chlorosis it caused. Ptr ToxB was purified from Alg-H2 culture filtrate, following essentially the same protocol as reported earlier for its purification from race 5 isolate Alg 3-24. The presence of a toxic principle, with the same specificity as Ptr ToxB, was also demonstrated in 92-171R5 culture filtrate.

3.2 Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera* tritici-repentis (Died.) Shoem.), the causal agent of tan spot, is an important foliar pathogen of wheat occurring worldwide (Hosford 1982). Tan spot is characterized by two distinct symptoms, tan necrosis and chlorosis. Isolates of the fungus were initially grouped into four pathotypes based on their ability to induce these symptoms in differential wheat genotypes. Isolates in pathotypes 1, 2, 3 and 4 cause, respectively, tan necrosis and chlorosis (nec^+chl^+), only tan necrosis (nec^+chl^-), only chlorosis (nec^-chl^+), or neither symptom (nec^-chl^-) (Lamari

and Bernier 1989b). The identification of isolates with a new virulence pattern led to the development of a race classification system (Lamari et al. 1995). Isolates are now grouped into races on the basis of their virulence on individual host differential genotypes. To date, five races have been reported: races 1 to 4 represent the previous pathotypes 1 to 4, while race 5 represents another chlorosis-inducing race, with a different virulence pattern than that of race 3 (Lamari et al. 1995). The advantage of this classification system is that the number of races that can be accommodated is limited only by the size and effectiveness of the wheat differential set.

An interesting aspect of the tan spot system is the production, by different isolates of the fungus, of host-specific toxins. The necrosis symptom caused by races 1 and 2 was shown to result from the production, by the pathogen, of Ptr ToxA (Lamari and Bernier 1989c), which was later characterized by various independent research groups (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Zhang et al. 1997). Ptr ToxA is a protein with a molecular mass of 13.2 kDa, and its coding region has been cloned (Ballance et al. 1996; Ciuffeti et al. 1997). Similarly, the chlorosis symptom induced by race 5 isolates was shown to be associated with the production of another toxin, termed Ptr ToxB (Orolaza et al. 1995). Ptr ToxB, which was recently purified and characterized, is also a small protein (6.61 kDa)(Strelkov et al. 1999). Genomic and cDNA clones of *ToxB*, the gene that codes for this toxin, have been obtained (Martinez et al. 2001; G.M. Ballance, *unpublished data*). Ptr ToxA was formerly known as Ptr necrosis toxin, Ptr toxin, and ToxA, while Ptr ToxB was formerly referred to as Ptr chlorosis toxin. In an effort to standardize toxin nomenclature in the tan spot system, the toxins were renamed Ptr ToxA and Ptr ToxB (Ciuffetti et al. 1998). Chlorosis in response to infection by races 1 and 3 isolates appears to be due to the

production of another host-specific toxin, designated Ptr ToxC, which has been partially purified (Effertz et al. 2002). In this paper, we compare the chlorosis symptoms produced by various isolates of *P. tritici-repentis* representing different races, and report the existence of a new race.

3.3 Materials and Methods

Terminology. The term "virulence" shall be used in this context to refer to a specific relationship between host and pathogen, where a race of the pathogen possesses one or more of the genes that allow it to establish a compatible relationship with a given cultivar. The terms "susceptibility" and "resistance" are used to describe the host reaction to the fungus, while "sensitivity" and "insensitivity" are used to describe the host reaction to the fungal toxins.

Plant Material. Wheat seeds were planted in clay pots, 15 cm in diameter, filled with a 2:1:1 (soil:sand:peat) soil mix (V:V:V). Seedlings were maintained in a growth room set at $22/18^0$C (day/night) with a 16 h photoperiod at a light intensity of 250 μmol/m^2/s. Plants were fertilized weekly and watered as required. The virulence pattern of the isolates was assessed using a differential set made up of two tetraploid and six hexaploid wheat genotypes (cvs. Glenlea, Katepwa, Erik, Salamouni, Coulter, lines 6B662, 6B365, 4B1149), with known reactions to the five currently described races of *P. tritici-repentis* (Lamari et al. 1995). Two cultivars or lines were planted per pot in separate clumps of 5-6 seeds each. All treatments were replicated three times. For comparison of chlorosis symptoms induced by the various isolates, wheat genotypes 'Katepwa', 6B662, 'Salamouni' and 6B365 were used. One cultivar or line was planted per pot as 5-6 evenly spaced seeds. All treatments were replicated four times, and the test was repeated twice.

Isolates. For assessment of virulence, seven isolates were used. Five of these, Asc 1, 86-124, D308, 90-2 and Alg 3-24 belong to races 1 to 5, respectively, and have been reported previously (Lamari and Bernier 1989b; Lamari et al. 1995). Two isolates not previously described, 92-171R5 and Alg-H2, were also examined. Isolate 92-171R5 was obtained from a hexaploid wheat field in Togo (Saskatchewan, Canada) on the Manitoba border in 1992, while Alg-H2 was isolated from a durum wheat field in Heliopolis, in eastern Algeria in 1993. For detailed comparison of chlorosis symptoms caused by infection with *P. tritici-repentis*, the four isolates D308, Alg 3-24, 92-171R5 and Alg-H2 were used.

Inoculation. Conidial inoculum was produced as described previously (Lamari and Bernier 1989a). Seedlings were inoculated at the 2-3 leaf stage with a suspension of 3500 conidia per mL, to which 10 drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per L were added. The suspension was applied to the seedlings using a deVilbis sprayer connected to an air line, at a pressure of approximately 67 kPa. After inoculation, plants were placed in a misting chamber for 24 h at 22/18^{0}C (day/night) with a 16 h photoperiod. Continuous leaf wetness was maintained by two ultrasonic humidifiers. Seedlings were then moved to a growth room bench and observed daily for symptom development.

Disease measurement. To assess virulence patterns on the differential set, seedlings were rated for disease development 6 d post-inoculation using the rating system of Lamari and Bernier (1989a).

For comparison of chlorosis symptom development among selected isolates, the percentage of total leaf area covered by chlorotic lesions was measured using Assess for Windows, an image analysis software developed by L. Lamari (2002). Leaves were harvested 6 to 7 d post-inoculation, placed in sealed plastic bags containing a piece of

moistened paper towel, and kept in a refrigerator or on ice until processed later the same day. Groups of 8-12 leaves were scanned on a flat-bed scanner (Visioneer PaperPort 6100B), and total leaf and lesion areas were measured using the image analysis software. All repetitions of each treatment were analyzed, and their means and standard deviations were calculated. This procedure was repeated for both runs of the experiment, and similar patterns were observed. Therefore, only the results from one run are presented.

Ptr ToxB production and bioassays. To test for Ptr ToxB toxin production by isolates 92-171R5 and Alg-H2, cultures of each isolate were grown on V8 potato dextrose agar (Lamari and Bernier 1989a), until they were 4 to 5 cm in diameter. Five plugs, 1 cm in diameter, were cut from each colony and transferred to 1 L Roux bottles containing 250 mL Fries medium amended with 0.1% yeast extract and possessing only 0.955 mM KH_2PO_4 and 1.49 mM K_2HPO_4 (Dhingra and Sinclair 1985). The cultures were incubated in the dark without agitation at 20^0C for 21 d.

Culture filtrates were collected by filtration through Whatman No. 1 filter paper and 0.45 μm cellulose nitrate filters. Several dilutions of the culture filtrates were made and assayed for toxic activity by infiltrating toxin-sensitive (cv. Katepwa) and insensitive (cv. Glenlea) seedlings at the two- to three-leaf stage with approximately 20 μL of sample, using a Hagborg (1970) device. The remaining culture filtrates were freeze-dried and stored at -20^0C until needed.

In the case of isolate 92-171R5, 1 g of freeze-dried culture filtrate was re-dissolved in 5 mL of 20 mM sodium acetate buffer (pH 4.6), and centrifuged at 17400 X g for 10 min. The supernatant was collected and dialyzed overnight against water, then tested for toxic activity.

Ptr ToxB was purified from isolate Alg-H2, using the same procedure previously described for purification of the toxin from race 5 isolate Alg 3-24 (Strelkov et al. 1999). The purity of the toxic fractions was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein estimation and electrophoresis. Protein concentration was estimated by the method of Lowry et al. (1951). Polyacrylamide gel electrophoresis was run under denaturing conditions with SDS, in a Mini-Protean II electrophoresis cell (Bio-Rad, Mississauga, Ontario, Canada), using a tris-tricine buffer system (Ausubel et al. 1996).

Antibody production. Polyclonal antibodies were raised against Ptr ToxB in rabbits at the NaH Biological Lab (Dugald, Manitoba, Canada). The immunoglobulin G (IgG) fraction was purified by affinity chromatography using a Protein A Antibody Purification Kit from Sigma-Aldrich (St. Louis, Missouri, USA), according to manufacturer's instructions. The purified IgG fraction was used as a stock preparation of primary antibody for Western blotting analysis.

Western blotting. Total soluble protein from concentrated culture filtrates of 21 d old cultures of isolates Alg 3-24, 92-171R5 and Alg-H2 were subjected to SDS-PAGE. SDS-PAGE mini-gels and nitrocellulose membranes (Amersham Pharmacia Biotech) were equilibrated for 15 min in transfer buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol). Proteins were then transferred from gels to nitrocellulose membranes at 100 V (260 mA) for 1 h in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). After transfer, blots were incubated overnight at 4^0C, with agitation, in TBS (137 mM NaCl, 2.7 mM KCl, and 25 mM Tris, pH 7.4) containing 1% (w/v) BSA. They were then quickly rinsed twice in TTBS (0.05% (v/v) Tween 20 in TBS), and incubated for

1 h with the IgG fraction of the Ptr ToxB antiserum, diluted 1/200 in antibody buffer (1% (v/v) BSA in TTBS). Blots were washed four times for 10 min each in TTBS, and then incubated with a 1/5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) in antibody buffer (Ausubel et al. 1996). Blots were washed again, ECL-detection reagents (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) were added as per manufacturer's instructions, and the blots were exposed to film.

3.4 Results

Virulence of isolates. All isolates tested were avirulent on cvs. Erik and Salamouni and line 4B1149 (Table 3.1). Isolate 92-171R5 possessed the same virulence pattern as race 5 isolate Alg 3-24. Both caused chlorosis on cv. Katepwa and line 6B662, necrosis on cv. Coulter, and were avirulent on all other lines/cvs. However, while the lesions formed on susceptible lines/cvs. in response to infection by isolate Alg 3-24 were generally rated 4-5 (most lesions coalescing), those formed in response to isolate 92-171R5 infection were smaller, usually rated as 3 (lesions generally not coalescing) (Table 3.1). Isolate Alg-H2 had a virulence pattern which combined the patterns of race 3 isolate D308 and race 5 isolate Alg 3-24 (Table 3.1). Like isolate Alg 3-24, isolate Alg-H2 was able to cause severe chlorosis on cv. Katepwa and line 6B662, as well as necrosis on cv. Coulter. Unlike isolate Alg 3-24, however, isolate Alg-H2 also caused chlorosis in line 6B365, in the same fashion as race 3 isolate D308 (Table 3.1).

Chlorosis symptom development. Chlorosis symptoms induced by isolates D308, Alg 3-24, 92-171R5 and Alg-H2 on selected wheat genotypes differed both quantitatively and

Table 3.1. Reaction of eight wheat genotypes to seven isolates of *Pyrenophora tritici-repentis* differing in ability to cause tan necrosis and chlorosis.

	Disease Rating[a]						
Cultivar	Race1	Race2	Race3	Race4	Race5		Race6
or line[b]	Asc1	86-124	D308	90-2	Alg3-24	92-171R5	Alg-H2
Glenlea	4-5N	4-5N	1-2	1	1-2	1	1-2
Katepwa	5N	5N	1-2	1	4-5C	3C	4-5C
6B662	1-2	1-2	1-2	1	4-5C	3C	4-5C
6B365	5C	1-2	5C	1	1-2	1	4-5C
Erik	1	1	1	1	1	1	1
Salamouni	1	1	1	1	1	1	1
4B1149	1	1	1	1	1	1	1
Coulter	4N	4N	4-5N	1	4-5N	3N	4-5N

[a]Plants were rated on a scale of 1-5 based on lesion type, where 1 and 2 represent resistance and 3-5 susceptibility. N = tan necrosis and C = chlorosis.

[b]All cultivars and lines listed are hexaploid except for line 4B1149 and cv. Coulter, which are tetraploid.

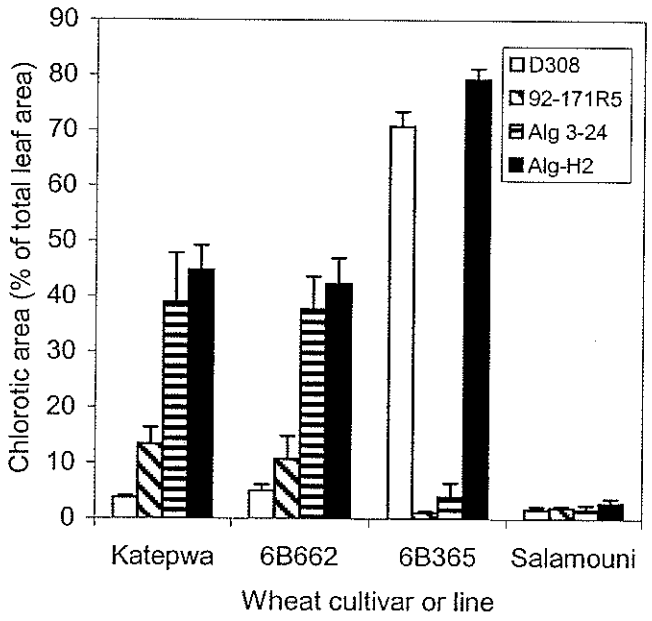
qualitatively. The chlorosis caused by isolates D308 and Alg-H2 on line 6B365 was more extensive and diffuse than that caused by isolates Alg 3-24 and Alg-H2 on cv. Katepwa and line 6B662 (Fig. 3.1); more than 70% of total leaf area was chlorotic in line 6B365 in response to infection by isolates D308 or Alg-H2, while approximately 40% of total leaf area was chlorotic in cv. Katepwa and line 6B662 in response to infection by isolates Alg 3-24 or Alg-H2 (Fig. 3.2). Isolate 92-171R5 caused substantially lower amounts of chlorosis (approximately 17%) on cv. Katepwa and line 6B662 than did isolates Alg 3-24 and Alg-H2 (Fig. 3.2). Nevertheless, chlorosis induced by isolate 92-171R5 resembled that caused by isolates Alg 3-24 and Alg-H2, although less severe (Fig. 3.1). No chlorosis was produced by any of the four isolates on resistant cvs. Salamouni or Erik (Table 3.1, Fig. 3.2).

Ptr ToxB production and bioassays. Culture filtrate of isolate Alg-H2 induced chlorosis at all dilutions tested in cv. Katepwa but not cv. Glenlea. Symptoms were strongest when undiluted culture filtrate was used, but strong symptoms were also observed when 1/5 dilutions were infiltrated into leaves of sensitive seedlings. There was distinct symptom development in response to infiltration with a 1/10 dilution of Alg-H2 culture filtrate, and faint symptoms developed after infiltration with 1/50 and 1/100 dilutions. No symptom development was observed on either cultivar as a result of infiltration with culture filtrate from isolate 92-171R5. However, when the filtrate was concentrated approximately 80-fold, it produced weak chlorosis symptoms when infiltrated into leaves of sensitive, but not insensitive, lines or cultivars (data not shown).

Toxic activity in the Alg-H2 culture filtrate was associated with protein precipitated at 25-80% ammonium sulfate saturation. The total soluble protein content of this fraction

Figure 3.1. Reactions of wheat cv. Katepwa and line 6B365 to infection by various chlorosis-inducing isolates of *P. tritici-repentis*. Shown are, from left to right, isolates Alg-H2, Alg 3-24 and 92-171R5 on line 6B365 and cv. Katepwa. Reactions on line 6B662 are not shown, but closely resembled those produced on cv. Katepwa. Isolate D308 (not shown) produced symptoms similar to Alg-H2 on line 6B365, but was avirulent on Katepwa (Table 1).

Figure 3.2. Percentage of total leaf area turning chlorotic in various wheat cultivars or lines in response to infection by isolates D308, 92-171R5, Alg 3-24 and Alg-H2 of *P. tritici-repentis*. Leaves were harvested six to seven days post-inoculation, and total leaf and lesion areas were measured using imaging software. Error bars indicate the standard deviation from multiple repetitions of one run of the experiment.


90
80
70
60
50
40
30
20
10
0
Chlorotic area (% of total leaf area)
D308
92-171R5
Alg 3-24
Alg-H2
Katepwa
6B662
6B365
Salamouni
Wheat cultivar or line

represented 46.1% of that present in the concentrated culture filtrate. The precipitate was re-dissolved, desalted and loaded onto a CM-S C25 column, where toxic activity eluted at approximately 300 mM NaCl (data not shown). The toxic fractions (representing 1.71% of the total soluble protein in the culture filtrate) were pooled, dialyzed, concentrated, and re-chromatographed on a FPLC MonoS HR 5/5 column. The linear salt gradient eluted three major peaks (labelled I, II and III), the first of which also possessed a shoulder peak (Ii) (Fig. 3.3). Toxic activity was only associated with peak I, which was eluted at approximately 50 mM NaCl and collected as two fractions (A and B). SDS-PAGE of fraction A revealed only one Coomasie blue stained band of approximately 6 kDa, which corresponded to Ptr ToxB (Strelkov et al., 1999) (Fig. 3.4, lane 2). Fraction B possessed both the 6 kDa band, plus a weaker, contaminating band approximately 27 kDa in size (data not shown). The pure Ptr ToxB recovered accounted for 0.0207% of the total soluble protein in the crude culture filtrate.

Western blotting. Western analysis of total soluble protein from concentrated culture filtrates of 21 d old cultures of isolates Alg 3-24, 92-171R5 and Alg-H2 revealed that the IgG fraction of rabbit Ptr ToxB antisera reacted with a protein approximately 6 kDa in size, corresponding to Ptr ToxB, in isolates Alg 3-24 and Alg-H2 (Fig. 3.5, lanes 1 and 3). Band intensities suggested that levels of toxin were similar in culture filtrates of these two isolates. However, no band corresponding to Ptr ToxB was observed in 21 d old 92-171R5 culture filtrate (Fig. 3.5, lane 2).

3.5 Discussion

Isolate Alg-H2, obtained from eastern Algeria, possesses a host range different from that of all other previously described races. Like isolates from race 5, Alg-H2 is able to

Figure 3.3. MonoS chromatography of Ptr ToxB from fungal isolate Alg-H2. Fractions showing toxic activity after CM-S C-25 chromatography were pooled and loaded onto a MonoS HR 5/5 column. The column was eluted with a linear gradient of 0 to 250 mM NaCl in 20 mM NaAc (pH 4.6) over 25 column volumes. The eluate was monitored at 280 nm and fractions were bioassayed for toxic activity. Toxic activity was only associated with peak I, collected as two fractions, A and B (indicated on figure). Fraction A contained pure toxin.

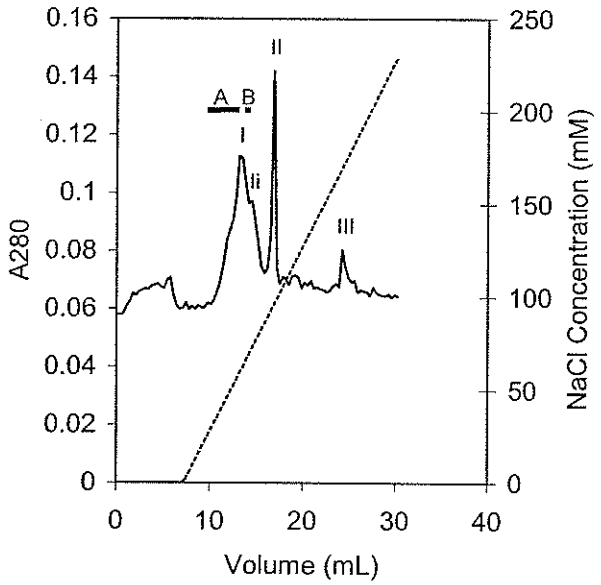

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
A280
250
200
150
100
50
0
NaCl Concentration (mM)
0
10
20
30
40
Volume (mL)
A
B
I
II
III

Figure 3.4. SDS-polyacrylamide gel electrophoresis of pure Ptr ToxB from isolates Alg 3-24 and Alg-H2 of *P. tritici-repentis*. Lane 1: molecular weight markers (10 μg of total protein). Lane 2: Pure Ptr ToxB (7.1 μg) purified from isolate Alg-H2. Lane 3: Pure Ptr ToxB (0.54 μg) purified from isolate Alg 3-24. Bands were stained with Coomassie blue G-250.

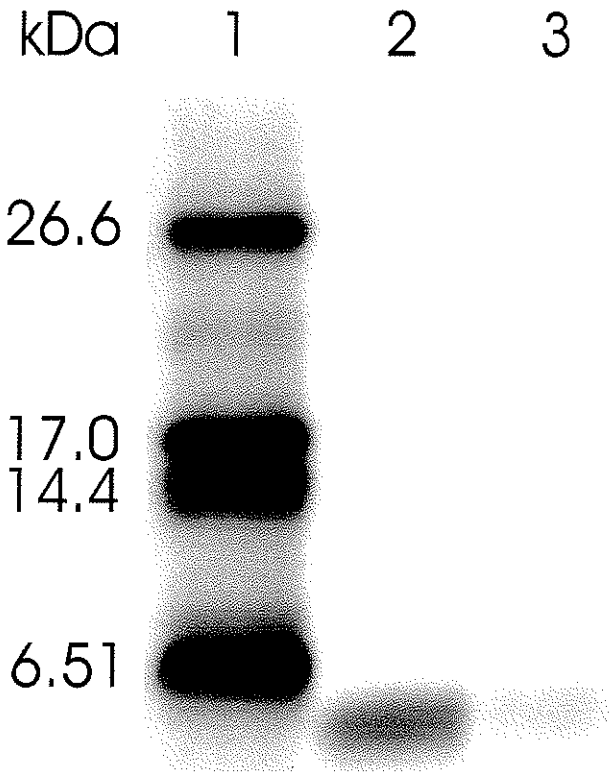
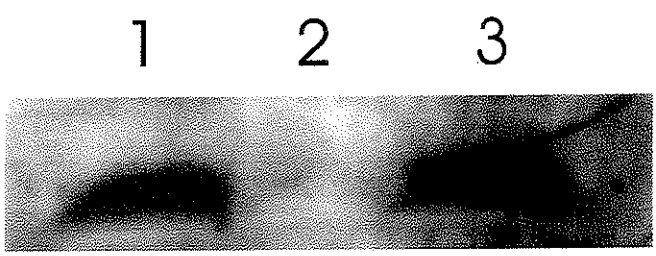

kDa
1
2
3
26.6
17.0
14.4
6.51

Figure 3.5. Western blot of concentrated culture filtrate from 21 d old cultures of isolates Alg 3-24 (lane 1), 92-171R5 (lane 2) and Alg-H2 (lane 3). One-hundred μg of protein from each isolate were run on a SDS-PAGE minigel and transferred to a nitrocellulose membrane. The blot was incubated with a 1/200 dilution of the IgG fraction of Ptr ToxB antisera, then with a 1/5000 dilution of horse radish peroxidase-conjugated secondary antibody. ECL-detection reagents were added as described in the Materials and Methods, and the blot was exposed to film.


1
2
3

induce chlorosis on wheat cv. Katepwa and line 6B662. However, it also induces chlorosis on 6B365, the wheat line used to differentiate race 3. Thus, the virulence pattern of Alg-H2 appears to be a combination of the patterns produced by races 3 and 5. Given the unique set of reactions produced by Alg-H2 on 'Katepwa', 6B662 and 6B365, we propose that it be classified as a new race, and be designated race 6.

The other novel isolate described in this study, 92-171R5, possesses the same host range as race 5 isolates. Although this isolate can be classified as belonging to race 5, it is much less aggressive than other race 5 isolates, as revealed by the size of the lesions (Table 3.1) and the extent of the chlorosis it caused (Fig. 3.1 and Fig. 3.2).

Ptr ToxB was shown to be a pathogenicity factor (Orolaza et al., 1995) required by the tan spot fungus to invade susceptible host tissue. This toxin was initially identified and purified from race 5 isolate Alg 3-24 (Orolaza et al. 1995; Strelkov et al. 1999). In the present study, we purified a protein from Alg-H2 culture filtrate that resembles Ptr ToxB in its physical/chemical behaviour during purification (Fig. 3.3 and Fig. 3.4). Furthermore, this protein reacts with the IgG fraction of polyclonal antisera raised against Ptr ToxB (Fig. 3.5), and is in all likelihood identical to this toxin. The ability of Alg-H2 to infect wheat cv. Katepwa and line 6B662 is therefore apparently due to its production of Ptr ToxB.

Purification of Ptr ToxB from cultures of isolate 92-171R5 was more problematic. The isolate appears to produce less toxin than Alg 3-24 and Alg-H2, as was evident from the fact that culture filtrate of 92-171R5 had to be concentrated approximately 80-fold before it could cause the typical chlorosis symptoms. This made it impossible to trace toxic activity during the various steps of the purification protocol, making it difficult to purify. However, the fact that concentrated 92-171R5 culture filtrate selectively induced chlorosis on cv.

Katepwa and line 6B662 suggested that the toxin is being produced. Nevertheless, no toxin band was visible in Western blots of total soluble protein from 21 d old culture filtrate of 92-171R5. Perhaps the level of Ptr ToxB produced in cultures of this isolate is too low to be detectable by Western blotting. Given the very high specific activity of the toxin (Strelkov et al. 1999), only trace amounts of it would be necessary to induce weak symptoms. However, it is not clear that production of Ptr ToxB is as low in vivo; symptoms produced in infections of susceptible wheat by isolate 92-171R5, while not as severe as those produced by other race 5 isolates, are nevertheless distinct (Fig. 3.1). Other studies are now in progress to examine Ptr ToxB production by different isolates of *P. tritici-repentis* in vivo. Nonetheless, lower amounts of toxin may at least partly account for the weaker symptoms caused by 92-171R5 upon infection of susceptible hosts (Table 3.1, Fig. 3.1 and Fig. 3.2). Moreover, it is possible that the toxic principle produced by 92-171R5, while related to Ptr ToxB, is not identical to it. Thus, the weaker symptoms observed may not be solely a result of lower toxin production, but could also be related to differences in sequence or structure, leading to a lower activity. This possibility is now being investigated.

Isolate Alg-H2 was also able to infect and produce symptoms on wheat line 6B365, much like a race 3 isolate (Table 3.1, Fig. 3.1 and Fig. 3.2). This suggests that Alg-H2 may produce Ptr ToxC in addition to Ptr ToxB. Ptr ToxC is apparently produced by races 1 and 3 isolates and has been partially purified from one such isolate (Effertz et al. 2002). By producing both Ptr ToxB and Ptr ToxC, Alg-H2 may have expanded its host range to include hosts sensitive to either of these toxins. The chlorosis symptoms produced on wheat line 6B365, in response to infection by race 3 isolates or Alg-H2, are more extensive and diffuse than those produced by race 5 isolates on cv. Katepwa or line 6B662 (Fig. 3.1 and

Fig. 3.2). This is consistent with the fact that while Ptr ToxB is, at 6.61 kDa, relatively large for a toxin, Ptr ToxC appears to be a smaller molecule (<2 kDa, Meinhardt et al. 1997) which may diffuse more readily through infected leaves.

The race 5 isolate reported here, 92-171R5, was collected in 1992 from a hexaploid wheat plant on the Manitoba-Saskatchewan border. Since then, race 5 isolates have only been reported once in North America (Ali and Francl 1999); all other race 5 isolates have been collected in Algeria (Lamari et al. 1995). Interestingly, 92-171R5 is the only race 5 isolate ever found on hexaploid rather than durum wheat. Furthermore, the race 6 isolate described in this paper serves as a link between the various races by its production of both Ptr ToxB and (putatively) Ptr ToxC. Isolates from races 1 and 2 produce Ptr ToxA (Lamari and Bernier 1989c), while race 5 isolates produce Ptr ToxB (Orolaza et al. 1995). Race 1 isolates also produce Ptr ToxC, as do isolates from race 3 (Effertz et al. 2002; L. Lamari, *unpublished data*). However, prior to the identification of race 6, isolates producing Ptr ToxB were not known to produce any other toxin. We speculate that we now have, in race 6, isolates capable of synthesizing both Ptr ToxB and Ptr ToxC. This combination would establish a continuum of toxin production among the races, with different races differing in their abilities to produce different toxins, and with some having particular toxins in common.

4.0 OCCURRENCE AND EXPRESSION OF *TOXB* AMONG RACES OF *PYRENOPHORA TRITICI-REPENTIS*

4.1 Abstract

Tan spot, caused by the fungus *Pyrenophora tritici-repentis*, is a major disease of wheat worldwide. Chlorosis in response to infection by races 5 and 6 of the pathogen develops as a result of their production of the host-specific toxin Ptr ToxB. The occurrence of the *ToxB* gene, which codes for Ptr ToxB, was investigated among isolates representing all known races of the fungus. A form of the gene was found to be present in races 3, 4, 5 and 6, but absent in races 1 and 2. Although Northern analysis revealed the presence of Ptr ToxB mRNA in total RNA extracted from races 5 and 6 isolates Alg 3-24 and Alg-H2, the technique was not sensitive enough to detect it in RNA from the other races. Using RT-PCR, however, the presence of Ptr ToxB mRNA was also confirmed in total RNA from ungerminated spores of isolates D308 (race 3), 90-2 (race 4), and 92-171R5 (a low aggressiveness race 5 isolate). PCR-products were cloned and sequenced, and the cDNA sequences coding for mature toxin were found to be 100% homologous among all isolates except 90-2, which exhibited only 86% homology with the others.

4.2 Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoemaker, causes tan spot, an important foliar disease of wheat throughout the world. The incidence of this stubble-borne disease has increased in recent decades, due to the adoption of conservation tillage techniques by farmers (Hosford 1982; Rees and Platz 1992). Tan spot is associated with the development of two distinct

symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier 1989a, 1989b). Isolates of *P. tritici-repentis* are classified into races based on their ability to cause these symptoms on a wheat differential set (Lamari et al. 1995). To date, six races of the fungus have been identified (Lamari et al. 1995; Strelkov et al. 2002).

Necrosis in hexaploid wheat lines or cultivars results from the production, by races 1 and 2 isolates of *P. tritici-repentis*, of a host-selective toxin (HST) termed Ptr ToxA (Lamari and Bernier 1989c). The mature form of Ptr ToxA is a 13.2 kDa protein, coded for by a single copy gene (*ToxA*) which has been cloned by two independent research groups (Ballance et al. 1996; Ciuffetti et al. 1997). Several lines of evidence, including the fact that Ptr ToxA possesses a host-specificity identical to that of the isolates from which it is obtained, as well as the transformation of a nonpathogenic isolate with *ToxA* (Ciuffetti et al. 1997), strongly suggest that Ptr ToxA is an important factor in the development of the tan spot disease (Tomás and Bockus 1987; Lamari and Bernier 1991; Ciuffetti et al. 1997).

Chlorosis in response to infection by races 5 and 6 isolates of *P. tritici-repentis* results from their production of a second HST, designated Ptr ToxB (Orolaza et al. 1995; Strelkov et al. 1999; Strelkov et al. 2002). This toxin causes chlorosis by a mechanism involving chlorophyll photo-oxidation, possibly as a result of an inhibition of photosynthesis (Strelkov et al. 1998). Like Ptr ToxA, Ptr ToxB appears to be a primary determinant of pathogenicity in the wheat-*P. tritici-repentis* interaction (Orolaza et al. 1995; Strelkov et al. 1999). Also like Ptr ToxA, Ptr ToxB is proteinaceous in nature; Strelkov et al. (1999) purified and characterized the toxin protein from race 5 isolate Alg 3-24. They determined the mass of Ptr ToxB to be 6.61 kDa, and predicted that it is 63

amino acids long (Strelkov et al. 1999). Furthermore, Strelkov et al. (1999) sequenced 29 of 30 N-terminal amino acid residues. G.M. Ballance (*unpublished data*) obtained a 417 bp cDNA of Ptr ToxB from isolate Alg 3-24, using the protein sequence information (Fig. 4.1). Martinez et al. (2001) cloned the region coding for Ptr ToxB (*ToxB*), utilizing the same protein sequence information, from race 5 isolate DW7. They found that *ToxB* codes for an 87 amino acid residue protein, including a 23 residue signal peptide, which is cleaved off to give a mature protein 64 residues in length and 6.5 kDa in mass (Martinez et al. 2001). In the present study, we investigated the occurrence and expression of the *ToxB* gene among different races of *P. tritici-repentis*, cloned their Ptr ToxB cDNA, and compared the different deduced protein products.

4.3 Materials and Methods

Isolates and fungal cultures. Isolates Asc1, 86-124, D308, 90-2, Alg 3-24 and Alg-H2 belong to races 1 to 6, respectively, of *P. tritici-repentis* and have been previously described (Lamari and Bernier 1989b; Lamari et al. 1995; Strelkov et al. 2002). Isolate 92-171R5 is a recently reported, low aggressiveness race 5 isolate (Strelkov et al. 2002). Mycelium from the various isolates was produced as described earlier (Lamari and Bernier 1989a; Strelkov et al. 1999). Liquid cultures were incubated in the dark, with agitation at 20^0C, and harvested at 3, 6 or 9 d post-inoculation. Mycelial mats were separated from the culture filtrates by vacuum filtration, frozen in liquid nitrogen and lyophilized. For production of ungerminated spores, conidia were produced as previously described (Lamari and Bernier 1989a), harvested, pooled, frozen in liquid nitrogen, and freeze-dried. All material was stored at -20^0C until processed.

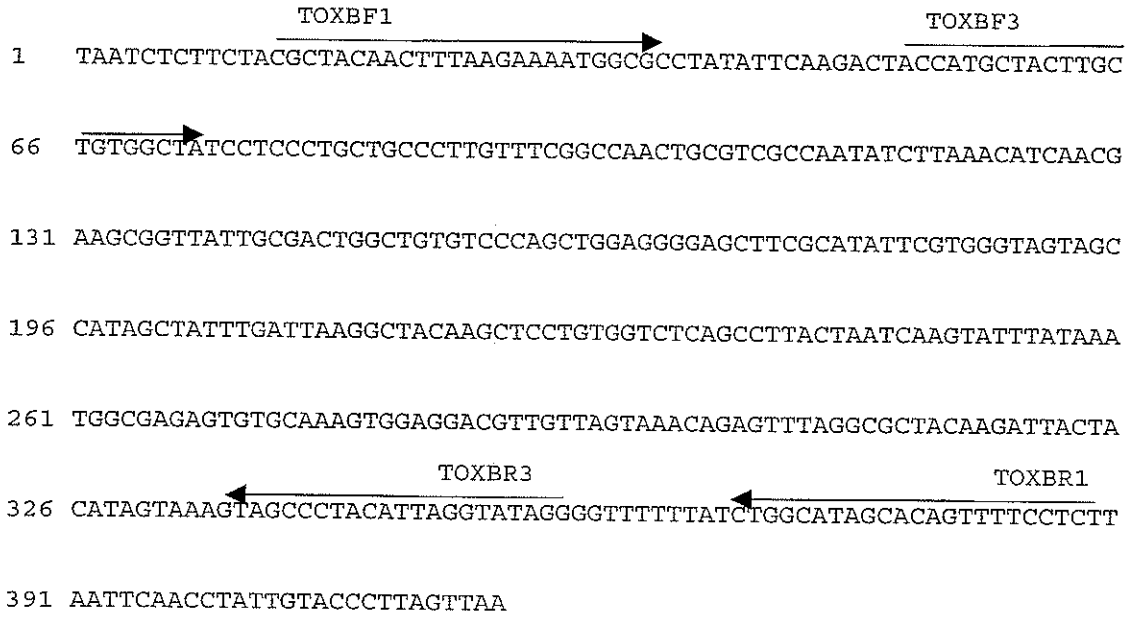


Figure 4.1. A 417 bp cDNA of Ptr ToxB from race 5 isolate Alg 3-24 of *P. tritici-repentis*. The cDNA was obtained by G.M. Ballance (*unpublished data*). It was radiolabeled and used as a probe in Southern and Northern blotting analyses. Arrows indicate the position and sequence of the template-specific portions of primers used for PCR.

DNA isolation and Southern blotting analysis. Genomic DNA was extracted from mycelia of 6 d old liquid cultures of *P. tritici-repentis* using cetyltrimethylammonium bromide (CTAB) following established protocols (Ausubel et al. 1996). Southern analysis was performed as described by Sambrook et al. (1989). DNA was quantified using UV absorption spectroscopy, and 5 µg genomic DNA from each isolate was digested to completion with EcoRI and separated on a 0.8% agarose gel. Separated DNA was transferred to Hybond-N^+ membranes (Amersham Canada, Oakville, Ontario) using a VacuGeneXL Vacuum Blotting System (Pharmacia Biotech, Baie d'Urfe, Quebec), according to manufacturer's instructions. Hybridizations were carried out at 62^0C, and membranes were probed with a full-length, radiolabeled cDNA of Ptr ToxB from isolate Alg3-24 (Fig. 4.1). Probe DNA (25 ng) was labeled with [α-^{32}P]dATP (NEN Life Science Products, Boston, MA) using random hexanucleotide primers according to the method of Feinberg and Vogelstein (1983). After washing, labeled membranes were exposed to Kodak XAR-5 film (Eastman Kodak Company, Rochester, NY) with intensifying screens at -70^0C for varying periods of time.

RNA isolation and Northern blotting analysis. Total RNA was isolated from ungerminated spores and 3, 6, and 9 d old mycelial mats of *P. tritici-repentis* according to the method of Mohapatra et al. (1987). RNA was quantified by UV absorption spectroscopy and size-fractionated on 1% agarose/formaldehyde gels as described by Chomczynski and Sacchi (1987). Ten μg of denatured total RNA were loaded per sample and transferred to Zeta-Probe membranes (Bio-Rad Laboratories (Canada), Mississauga, Ontario) by vacuum blotting. Loading equivalence and RNA integrity were confirmed by ethidium bromide staining of gels prior to transfer. Hybridizations were conducted

over 16 h at 43^{0}C, in a mixture containing 500mM $NaPO_4$ buffer, 2.5M NaCl, 7% (w/v) SDS and 50% (v/v) formamide. Membranes were probed with a radiolabeled cDNA of Ptr ToxB (Fig. 4.1). Probe DNA (50 ng) was labeled with [α-^{32}P]ATP according to the random primer procedure described by Feinberg and Vogelstein (1983). Washed blots were exposed at –70^{0}C to Kodak XAR-5 films with intensifying screens for several days.

RT-PCR of fungal RNA. Total RNA (5 μg) isolated from ungerminated spores was reverse-transcribed with the Thermoscript RT-PCR System (Invitrogen Life Technologies, Burlington, Ontario), using the oligo(dT)$_{20}$ primer, as per manufacturer's instructions. Amplification was carried out following the GATEWAY Cloning Technology Adapter PCR Protocol (Invitrogen Life Technologies), to allow cloning of the PCR-products into the pDONR201 vector via the site-specific recombination system of bacteriophage lambda. Therefore, in the first step of this two-step PCR protocol, template-specific forward and reverse primers were designed with 12 bases of the *att*B1 and *att*B2 adapter primers at their 5'-ends, respectively. The template-specific portions of the primers were based on the Ptr ToxB cDNA sequence obtained by G.M. Ballance (Fig. 4.1). Two sets of primers were utilized (Fig. 4.1): (1) TOXBF1 (forward primer, 5'-12b *att*B1-GCTACAACTTTAAGAAAATGGGC-3') and TOXBR1 (reverse primer, 5'-12b *att*B2-GAGGAAAACTGTGCTATGCCA-3'), and (2) TOXBF3 (forward primer, 5'-12b *att*B1-CCATGCTACTTGCTGTGGCT-3') and TOXBR3 (reverse primer, 5'-12b *att*B2-CTATACCTAATGTAGGGCTAC-3'). The first PCR-step included an initial denaturation (95^{0}C for 2 min), followed by 10 cycles of denaturation (94^{0}C for 15 s), annealing (30 s at 53^{0}C for both sets of primers), and extension (68^{0}C for 1 min). After the first PCR, 10 μL from each of the reactions were transferred to 40 μL PCR mixtures

containing the full-length *att*B1 and *att*B2 adapter primers, and PCR was conducted as outlined in the manufacturer's protocol. Based on the cDNA of isolate Alg 3-24 (Fig. 4.1) and the sequence information from Martinez et al. (2001), the anticipated size of the amplified fragment produced by primers TOXBF1 and TOXBR1 was 433 bp, while that produced by primers TOXBF3 and TOXBR3 was 361 bp.

Cloning and sequencing of PCR-products. Ptr ToxB cDNA PCR-products were cloned into the pDONR201 plasmid using the GATEWAY PCR-Cloning System (Invitrogen Life Technologies), in accordance with manufacturer's instructions. Library efficiency DH5α competent cells (Invitrogen Life Technologies) were then transformed with Ptr ToxB cDNA (from the various isolates) in pDONR201, as per instructions for the GATEWAY PCR-Cloning System. Plasmids were isolated with a QIAGEN Plasmid Midi Kit (QIAGEN, Mississauga, Ontario), and the purified plasmids sent for sequencing to the PBI/NRC Sequencing Laboratory (Saskatoon, Saskatchewan). Nucleotide sequencing was performed on a Cetus DNA Thermal Cycler (Perkin Elmer, Boston, MA) using a Prism Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), with SeqL-A and SeqL-B sequencing primers (Invitrogen Life Technologies).

Image analysis. Gels and blots were analyzed on a Fluor-S MultiImager, using Quantity One Software (Bio-Rad).

4.4 Results

Occurrence of *ToxB*. Southern analysis was conducted to determine the presence or absence of the *ToxB* gene among different isolates of *P. tritici-repentis*. A 417 bp cDNA of Ptr ToxB from isolate Alg 3-24 (Fig. 4.1) was radiolabeled and used as a probe to

hybridize genomic DNA digested with EcoRI. No hybridization signal was observed with DNA from races 1 and 2 isolates Asc1 and 86-124 (results not shown). However, a single hybridizing fragment, approximately 23 kb in length, was detected in DNA from race 3 isolate D308 (Fig. 4.2, lane 1). In addition, a hybridizing band of approximately 18 kb was observed in DNA from race 4 isolate 90-2 (Fig. 4.2, lane 2). Southern analysis of genomic DNA from isolates Alg 3-24 and Alg-H2, representing races 5 and 6, respectively, revealed the presence of four hybridizing fragments in each isolate (Fig 4.2, lanes 3 and 5). The four different fragments appeared to be of the same length in both isolates (approximately 25, 16, 11 and 7 kb). Two hybridizing fragments, 11 and 6 kb in size, were also detected in DNA from low aggressiveness race 5 isolate 92-171R5 (Fig. 4.2, lane 4).

Presence of Ptr ToxB mRNA. To investigate whether or not transcription of *ToxB* occurs in those isolates possessing the gene, total RNA from 3, 6 and 9 d old mycelial mats, as well as from ungerminated spores, was extracted and subjected to Northern blotting analysis using the same probe as in the Southerns. A single hybridizing band was detected in mycelial mat total RNA from isolates Alg 3-24 and Alg-H2, at all time points tested, although the signal was strongest at 9 d for isolate Alg 3-24 and at 6 d for Alg-H2 (Fig. 4.3). Nevertheless, this finding was consistent with the presence of Ptr ToxB mRNA in the mycelia of these isolates. No signal was observed in mycelial mat total RNA from isolates D308, 90-2 and 92-171R5 at any time point (Fig. 4.3). Northern hybridization also revealed the presence of Ptr ToxB mRNA in the total RNA from ungerminated spores of isolates Alg 3-24 and Alg-H2, but the signals were much weaker

Figure 4.2. Southern blot analysis of genomic DNA from different isolates of *P. tritici-repentis*. Genomic DNA (5 μg) from isolates D308 (lane 1), 90-2 (lane 2), Alg 3-24 (lane 3), 92-171R5 (lane 4) and Alg-H2 (lane 5) was digested with EcoRI, separated on an agarose gel and blotted onto a Hybond-N^+ membrane. The blot was probed with a radiolabeled cDNA of Ptr ToxB from isolate Alg 3-24 (Fig. 4.1). No hybridization signal was observed with DNA from isolates Asc1 and 86-124 (not shown).

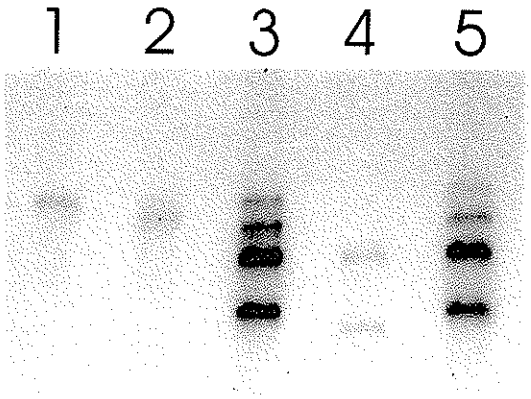
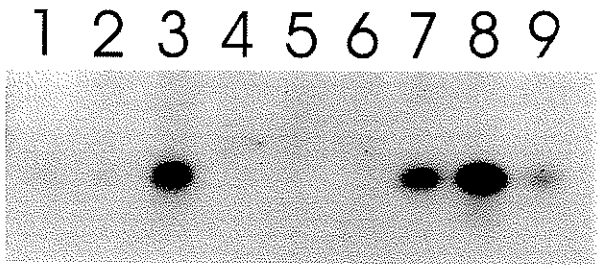

1
2
3
4
5

Figure 4.3. Northern blot analysis of total RNA extracted from mycelia of various *P. tritici-repentis* isolates at different time points. RNA was run on an agarose gel, blotted onto a Zeta-Probe membrane and probed with a radiolabeled cDNA of Ptr ToxB from isolate Alg 3-24 (Fig. 4.1). Each lane contained 10 μg total RNA extracted from 3, 6 and 9 d old cultures of: isolate Alg 3-24 (lanes 1-3, respectively), isolate 92-171R5 (lanes 4-6, respectively), and isolate Alg-H2 (lanes 7-9, respectively). No hybridizing bands were observed with total mycelial RNA from isolates D308 and 90-2 (not shown).


1 2 3 4 5 6 7 8 9

than observed in RNA from mycelial mats (Fig. 4.4). There appeared to be more Ptr ToxB mRNA in the total spore RNA of isolate Alg-H2 than Alg 3-24, as suggested by their respective band intensities (Fig. 4.4, lanes 4 and 5). As was the case with total RNA from their mycelia, no hybridizing bands were observed in the total RNA from ungerminated spores of isolates D308, 90-2 and 92-171R5 (Fig. 4.4, lanes 1, 2 and 3).

The failure to detect Ptr ToxB mRNA in the total RNA from isolates D308, 90-2 and 92-171R5 could be due to its complete absence, or alternatively, to its presence in levels too low to detect by conventional Northern blotting analysis. To distinguish between these two possibilities, we subjected total RNA from ungerminated spores to RT-PCR. As described in the Materials and Methods, total RNA was reverse-transcribed and then subjected to PCR using two sets of primers. The primer pair TOXBF1 and TOXBR1 amplified a product (between 400 and 500 bp) when used with spore cDNA from isolates Alg 3-24 and Alg-H2, but failed to amplify any visible product from isolates D308, 90-2 and 92-171R5 (Fig. 4.5A). Primers TOXBF3 and TOXBR3, which are internal to TOXBF1 and TOXBR1 on the Ptr ToxB cDNA sequence from isolate Alg 3-24 (Fig. 4.1), amplified a product (between 300 and 400 bp) in isolates D308, 90-2 and 92-171R5, as well as in isolates Alg 3-24 and Alg-H2 (Fig. 4.5B). The intensity of the bands obtained for Alg 3-24 and Alg-H2, when using primers TOXBF3 and TOXBR3, was greater than that obtained for isolates D308, 90-2 and 92-171R5 (Fig. 4.5B). The size of the products obtained with both primer sets corresponded well to the expected lengths of the amplified Ptr ToxB cDNA fragments (with the attB adapters at their 5' and 3' ends).

Figure 4.4. Northern blot analysis of total RNA extracted from ungerminated spores of different isolates of *P. tritici-repentis*. Total RNA (10 μg) from isolates D308 (lane 1), 90-2 (lane 2), 92-171R5 (lane 3), Alg 3-24 (lane 4) and Alg-H2 (lane 5) was run on an agarose gel, blotted onto a Zeta-Probe membrane, and probed with a radiolabeled cDNA of Ptr ToxB from isolate Alg 3-24 (Fig. 4.1).

1 2 3 4 5

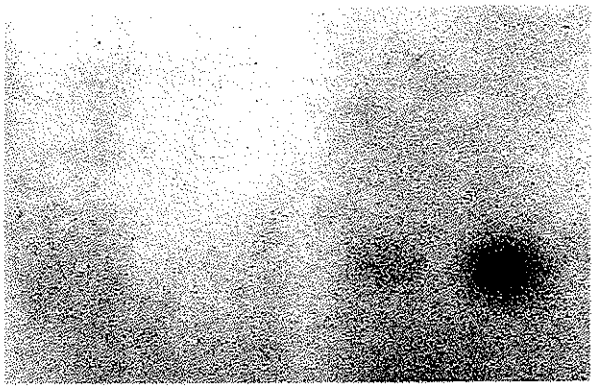
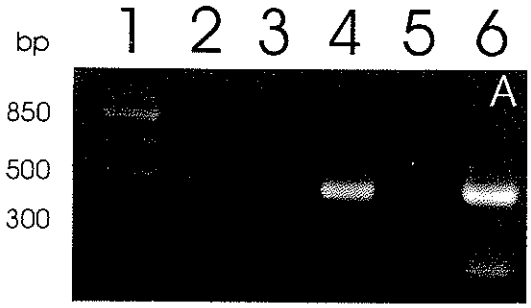
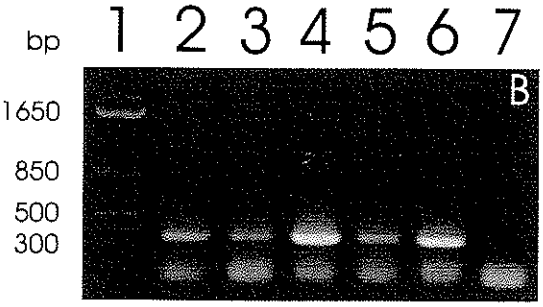


Figure 4.5. Ethidium bromide-stained gels of RT-PCR products from different *P. tritici-repentis* isolates, obtained using primers based on the Ptr ToxB cDNA sequence from isolate Alg 3-24 (Fig. 4.1). Total RNA (5 μg) from ungerminated spores of the various isolates was reverse-transcribed and then amplified using one of two sets of *ToxB*-based primers. After PCR, 10 μL of each reaction mixture were run on the gels. In gel A, the RT-PCR products obtained using primers TOXBF1 and TOXBR1 are shown. No product was observed for isolates D308 (lane2), 90-2 (lane 3), or 92-171R5 (lane 5). A product of between 400 and 500 bp can be seen for isolates Alg 3-24 (lane 4) and Alg-H2 (lane 6). In gel B, the RT-PCR products obtained with primers TOXBF3 and TOXBR3 are shown. A product of between 300 and 400 bp is seen for isolates D308 (lane 2), 90-2 (lane 3), Alg 3-24 (lane 4), 92-171R5 (lane 5) and Alg-H2 (lane 6). Lane 7 in gel B is a water control, and a DNA ladder was run in lane 1 of both gels.


bp
1 2 3 4 5 6
850
500
300
A


bp
1 2 3 4 5 6 7
1650
850
500
300
B

Nucleotide and deduced amino acid sequences. Utilizing the PCR-products synthesized with primers TOXBF1 and TOXBR1, clones of Ptr ToxB cDNA were obtained from isolates Alg 3-24 and Alg-H2 (Fig. 4.6). Since TOXBF1 and TOXBR1 produced no PCR-products from the reverse-transcribed total RNA of isolates D308, 90-2 and 92-171R5, the PCR-products created with primers TOXBF3 and TOXBR3 were used to obtain Ptr ToxB cDNA clones from these isolates (Fig. 4.6). The cDNA sequences obtained for isolates Alg 3-24 and Alg-H2 (GenBank accession nos. AF483831 and AF483830, respectively) are 375 bp long and 100% homologous (Fig. 4.6). They are also identical to the corresponding stretch of the *ToxB* sequence from isolate DW7 (Martinez et al. 2001), and the Ptr ToxB cDNA sequence previously obtained from isolate Alg 3-24 by G.M. Ballance (Fig. 4.1). The cDNA sequences from isolates D308 and 92-171R5 (accession nos. AF483833 and AF483834, respectively) are 303 bp long and 100% homologous to the corresponding section of the sequences from isolates Alg 3-24, Alg-H2 and DW7 (Fig. 4.6 and Martinez et al. 2001). The cDNA sequence from isolate 90-2 (accession no. AF483832) is 303 bp long and only 86% homologous to that of the other isolates. Numerous changes between this sequence and the rest are illustrated in Figure 4.6.

The deduced amino acid sequences of Ptr ToxB from the different isolates are illustrated in Figure 4.7. A Met and an Ala residue at the N-termini of the Alg 3-24 and Alg-H2 sequences are not shown, as they were encoded for by nucleotides sitting under the 3' end of the TOXBF1 primer. However, given the 100% homology between these two sequences and those of Ballance (Fig. 4.1) and Martinez et al. (2001), it would appear that these determinations were accurate. Thus, the first 23 residues from isolates

Figure 4.6. Nucleotide sequence comparisons of Ptr ToxB cDNAs from different isolates of *P. tritici-repentis*. Translation stop codons are indicated by three asterixes (***). Dashes (-) represent gaps introduced to align the sequences. Sequences are identical except where nucleotides are boxed in white. The GenBank accession numbers of the aligned sequences are: Alg 3-24, AF483831; Alg-H2, AF483830; D308, AF483833; 92-171R5, AF483834; 90-2, AF483832.

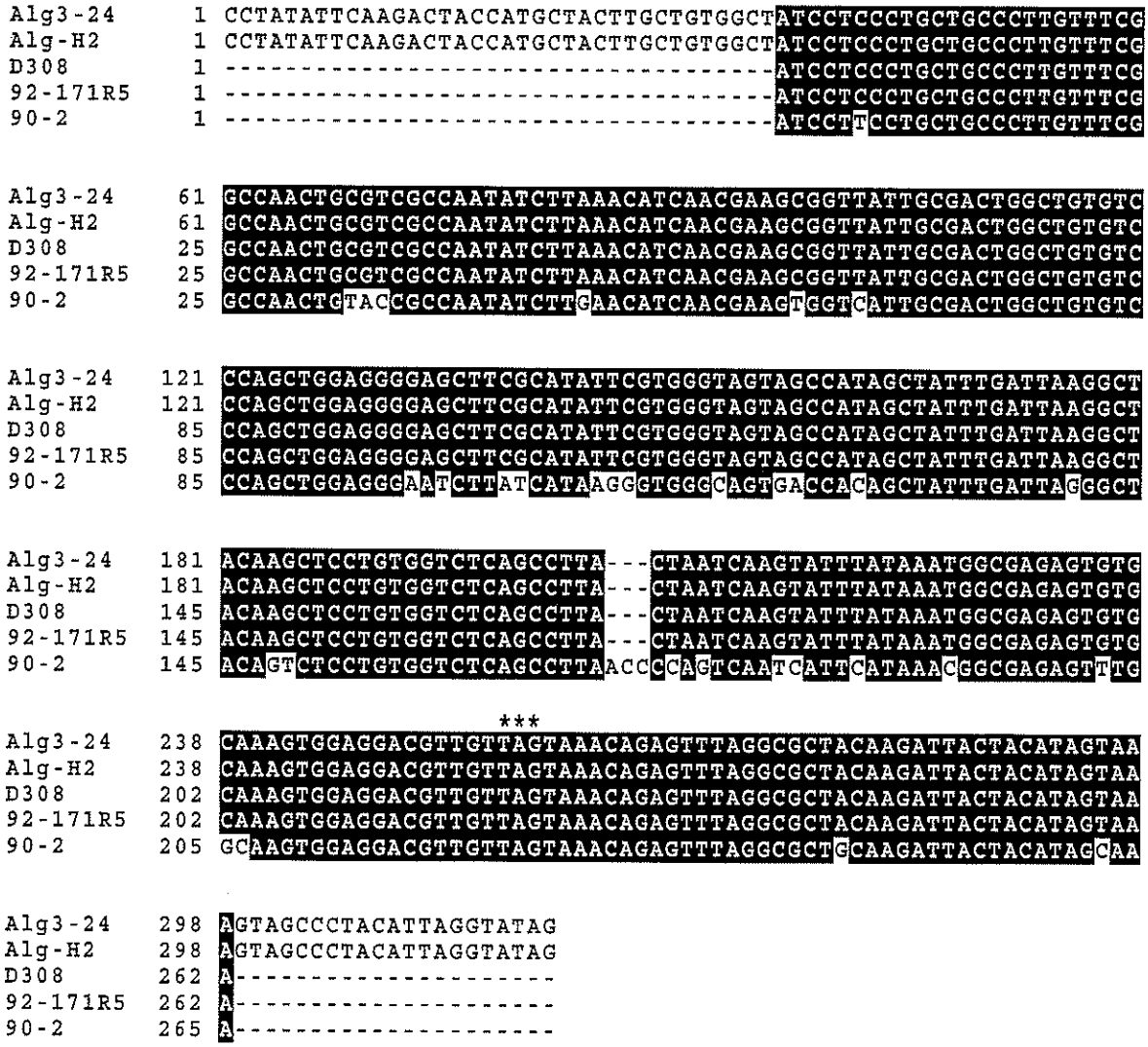
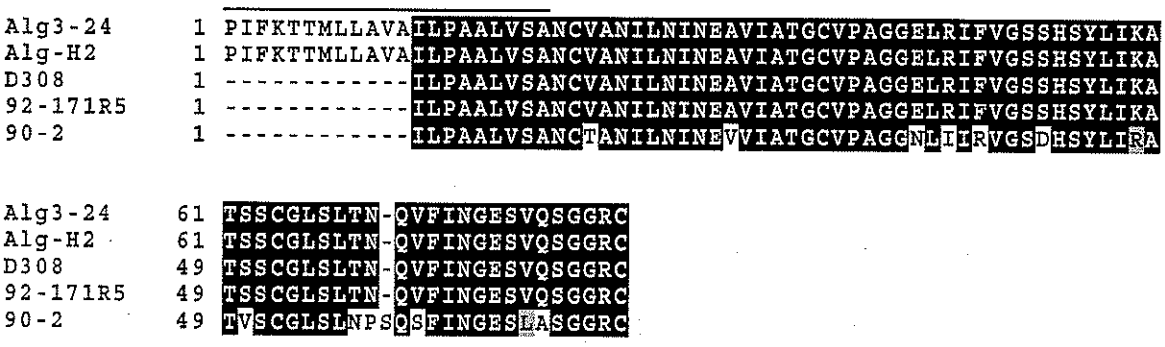

Alg3-24 1 CCTATATTCAAGACTACCATGCTACTTGCTGTGGCTATCCTCCCTGCTGCCCTTGTTTCG
Alg-H2 1 CCTATATTCAAGACTACCATGCTACTTGCTGTGGCTATCCTCCCTGCTGCCCTTGTTTCG
D308 1 ATCCTCCCTGCTGCCCTTGTTTCG
92-171R5 1 ATCCTCCCTGCTGCCCTTGTTTCG
90-2 1 ATCCTTCCTGCTGCCCTTGTTTCG
Alg3-24 61 GCCAACTGCGTCGCCAATATCTTAAACATCAACGAAGCGGTTATTGCGACTGGCTGTGTC
Alg-H2 61 GCCAACTGCGTCGCCAATATCTTAAACATCAACGAAGCGGTTATTGCGACTGGCTGTGTC
D308 25 GCCAACTGCGTCGCCAATATCTTAAACATCAACGAAGCGGTTATTGCGACTGGCTGTGTC
92-171R5 25 GCCAACTGCGTCGCCAATATCTTAAACATCAACGAAGCGGTTATTGCGACTGGCTGTGTC
90-2 25 GCCAACTGTACCGCCAATATCTTGAACATCAACGAAGTGGTCATTGCGACTGGCTGTGTC
Alg3-24 121 CCAGCTGGAGGGGAGCTTCGCATATTCGTGGGTAGTAGCCATAGCTATTTGATTAAGGCT
Alg-H2 121 CCAGCTGGAGGGGAGCTTCGCATATTCGTGGGTAGTAGCCATAGCTATTTGATTAAGGCT
D308 85 CCAGCTGGAGGGGAGCTTCGCATATTCGTGGGTAGTAGCCATAGCTATTTGATTAAGGCT
92-171R5 85 CCAGCTGGAGGGGAGCTTCGCATATTCGTGGGTAGTAGCCATAGCTATTTGATTAAGGCT
90-2 85 CCAGCTGGAGGGAATCTTATCATAAGGGTGGGCAGTGACCACAGCTATTTGATTAGGGCT
Alg3-24 181 ACAAGCTCCTGTGGTCTCAGCCTTA---CTAATCAAGTATTTATAAATGGCGAGAGTGTG
Alg-H2 181 ACAAGCTCCTGTGGTCTCAGCCTTA---CTAATCAAGTATTTATAAATGGCGAGAGTGTG
D308 145 ACAAGCTCCTGTGGTCTCAGCCTTA---CTAATCAAGTATTTATAAATGGCGAGAGTGTG
92-171R5 145 ACAAGCTCCTGTGGTCTCAGCCTTA---CTAATCAAGTATTTATAAATGGCGAGAGTGTG
90-2 145 ACAGTCTCCTGTGGTCTCAGCCTTAACCCCAGTCAATCATTCATAAACGGCGAGAGTTTG
***
Alg3-24 238 CAAAGTGGAGGACGTTGTTAGTAAACAGAGTTTAGGCGCTACAAGATTACTACATAGTAA
Alg-H2 238 CAAAGTGGAGGACGTTGTTAGTAAACAGAGTTTAGGCGCTACAAGATTACTACATAGTAA
D308 202 CAAAGTGGAGGACGTTGTTAGTAAACAGAGTTTAGGCGCTACAAGATTACTACATAGTAA
92-171R5 202 CAAAGTGGAGGACGTTGTTAGTAAACAGAGTTTAGGCGCTACAAGATTACTACATAGTAA
90-2 205 GCAAGTGGAGGACGTTGTTAGTAAACAGAGTTTAGGCGCTGCAAGATTACTACATAGCAA
Alg3-24 298 AGTAGCCCTACATTAGGTATAG
Alg-H2 298 AGTAGCCCTACATTAGGTATAG
D308 262 A
92-171R5 262 A
90-2 265 A

Figure 4.7. Alignment of the deduced Ptr ToxB amino acid sequences from different isolates of *P. tritici-repentis*. The solid line indicates a putative signal peptide. Amino acid substitutions and insertions are boxed in white or grey. Grey boxes signify a substitution with a similar amino acid.


Alg3-24 1 PIFKTTMLLAVAILPAALVSANCVANILNINEAVIATGCVPAGGELRIFVGSSHSYLIKA
Alg-H2 1 PIFKTTMLLAVAILPAALVSANCVANILNINEAVIATGCVPAGGELRIFVGSSHSYLIKA
D308 1 ILPAALVSANCVANILNINEAVIATGCVPAGGELRIFVGSSHSYLIKA
92-171R5 1 ILPAALVSANCVANILNINEAVIATGCVPAGGELRIFVGSSHSYLIKA
90-2 1 ILPAALVSANCTANILNINEVVIATGCVPAGGNLLIRVGSDHSYLIRA
Alg3-24 61 TSSCGLSLTN-QVFINGESVQSGGRC
Alg-H2 61 TSSCGLSLTN-QVFINGESVQSGGRC
D308 49 TSSCGLSLTN-QVFINGESVQSGGRC
92-171R5 49 TSSCGLSLTN-QVFINGESVQSGGRC
90-2 49 TVSCGLSLNPSQSFINGESLASGGRC

Alg 3-24 and Alg-H2 appear to constitute a signal peptide, as do the first 9 residues of the sequences from isolates D308, 90-2 and 92-171R5 (Nielsen et al. 1997). After cleavage of the signal peptide, mature Ptr ToxB from isolates Alg 3-24, Alg-H2, D308 and 92-171R5 seems to be 64 amino acids long with a mass of 6.54 kDa. These values are similar to the 63 amino acid length and 6.61 kDa mass predicted by Strelkov et al. (1999), and identical to those found by Martinez et al. (2001). The nucleotide differences found in the Ptr ToxB cDNA of isolate 90-2 are reflected in the amino acid composition of the protein for which it codes. Relative to the protein sequences from the other isolates, there are 13 amino acid substitutions and one insertion in mature Ptr ToxB from isolate 90-2, and only two of the substitutions are with similar amino acids (Fig.4.7). The protein is 65 amino acids long, while its predicted mass is 6.62 kDa. Database searches revealed no sequences (with the exception of *ToxB* from isolate DW7) with significant similarity to the Ptr ToxB cDNA or protein sequences obtained from isolates D308, 90-2, Alg 3-24, 92-171R5 or Alg-H2.

4.5 Discussion

Studies with Ptr ToxA revealed that only tox^+ isolates of *P. tritici-repentis* possess the *ToxA* gene (Ballance et al. 1996; Ciuffetti et al. 1997). Furthermore, Ptr ToxA functions as a pathogenicity factor in all isolates in which it is produced (Lamari and Bernier 1991; Ballance et al. 1996; Ciuffetti et al. 1997), and appears to be identical or nearly identical in all of these isolates (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995; Ballance et al. 1996; Ciuffetti et al. 1997; Zhang et al. 1997). It is apparent from the results presented here that this is not the case with Ptr ToxB. Southern hybridization showed that the *ToxB* gene is present in many races that were not known to

produce the toxin (Fig. 4.2). Race 3 isolate D308 was hypothesized to produce Ptr ToxC, but not Ptr ToxB, while race 4 isolate 90-2 is avirulent and was thought to produce no toxins (Strelkov et al. 2002). On the other hand, the occurrence of *ToxB* in isolates Alg 3-24, 92-171R5 and Alg-H2 was expected. Isolates Alg 3-24 and Alg-H2 belong to races 5 and 6, respectively, and Ptr ToxB has been purified from the culture filtrates of both (Strelkov et al. 1999; Strelkov et al. 2002). Low aggressiveness race 5 isolate 92-171R5 is also believed to produce Ptr ToxB, albeit at much lower levels (Strelkov et al. 2002).

Previous studies also indicated that *ToxA* is a single copy gene (Ballance et al. 1996; Ciuffetti et al. 1997). In contrast, the results of this study suggest that there are multiple copies of *ToxB* in isolates Alg 3-24 and Alg-H2; four bands were observed in hybridizations of their genomic DNA after digestion with EcoRI (Fig. 4.2). Similarly, Southern blotting analysis of genomic DNA from isolate 92-171R5 revealed the presence of two hybridizing fragments (Fig. 4.2). However, only single copies of *ToxB* appear to be present in isolates D308 and 90-2, since just one hybridizing band was observed in DNA digests of each of these (Fig. 4.2).

Having established the occurrence of *ToxB* among isolates known to produce the toxin as well as those previously not thought to do so, it was important to see whether or not the gene is being transcribed. As expected, Northern blotting analysis revealed the presence of Ptr ToxB mRNA in total RNA from isolates Alg 3-24 and Alg-H2, although the expression profiles differed somewhat (Fig. 4.3 and Fig. 4.4). However, no hybridizing bands were observed in any of the other isolates, including 92-171R5, in which we would have expected some level of Ptr ToxB mRNA (Fig. 4.3 and Fig. 4.4). Therefore, RT-PCR was utilized to detect very low abundance transcripts. Using two

sets of primers, Ptr ToxB cDNA was amplified from isolates Alg 3-24, Alg-H2, 92-171R5, D308 and 90-2 (Fig. 4.5). Hence, at least trace quantities of Ptr ToxB or Ptr ToxB-like mRNA were present in the total RNA of these isolates. Not surprisingly, PCR-band intensities were strongest for isolates Alg 3-24 and Alg-H2 (Fig. 4.5), presumably due to the fact that there was more initial template. A PCR-product could be obtained from isolates D308, 90-2 and 92-171R5 using primers TOXBF3 and TOXBR3, but not TOXBF1 and TOXBR1 (Fig. 4.6). This observation suggests that the upstream and/or downstream cDNA sequences of Ptr ToxB from isolates D308, 90-2 and 92-171R5 differ substantially from those of isolates Alg 3-24 and Alg-H2, since primers TOXBF3 and TOXFR3 are internal to TOXBF1 and TOXBR1 on the Ptr ToxB cDNA sequence (Fig. 4.1). The differences in sequence may be reflected in the lower intensity of the bands observed in Southern blotting analysis of genomic DNA from isolates D308, 90-2 and 92-171R5 (Fig. 4.2).

Despite the possible upstream or downstream differences in the Ptr ToxB cDNA sequences from the various isolates, there was a high degree of homology with respect to the regions coding for the mature protein (Fig. 4.6 and Fig. 4.7). The homology between isolates D308, 92-171R5, Alg 3-24 and Alg-H2 was 100%. The only sequence that displayed any differences came from isolate 90-2, the homology of which was only 86%. Several possible explanations exist as to why, despite the fact that the region coding for mature Ptr ToxB among the various isolates is highly conserved, there is only weak toxin activity in isolate 92-171R5 and no activity in isolates D308 and 90-2. The most obvious explanation is related to levels of expression. Even in the culture filtrates of isolates Alg 3-24 and Alg-H2, Ptr ToxB is not very abundant (Strelkov et al. 1999;

Strelkov et al. 2002). Thus, if expression is lower in the other isolates, as suggested by the Northern blots and RT-PCR, it is possible that there are insufficient toxin quantities to produce any observable effect. In the case of 92-171R5, there is evidence for weak toxin activity (Strelkov et al. 2002), and this may reflect an intermediate level of *ToxB* expression for this isolate. This hypothesis is supported by hybridization studies with RT-PCR products, using radiolabeled Ptr ToxB cDNA from isolate Alg 3-24 (Fig. 4.1), in which the signal intensity observed for the 92-171R5-derived product was intermediate among the isolates (results not shown). Differences in the upstream promoter or enhancer regions for *ToxB* may explain the differences in gene expression among the isolates. In addition, the number of copies of the gene could be significantly impacting the amount of transcript. The lack of toxin activity in isolate 90-2 may be easier to explain. Not only does this isolate probably produce much less toxin, differences in the amino acid composition of its mature Ptr ToxB (Fig. 4.6 and Fig. 4.7) could also be reducing its specific activity.

Other explanations for the lack of toxin activity in isolates D308 and 90-2 are possible, including a deficiency in translation. While antibodies raised against Ptr ToxB were useful in detecting the protein in culture filtrates from isolates Alg 3-24 and Alg-H2 (Strelkov et al. 2002), they could not detect any Ptr ToxB in the culture filtrates or spore germination fluids from any of the other isolates (results not shown). However, this was expected due to the low titer of the antibodies that we were using; they failed to detect Ptr ToxB at quantities below 200 ng (results not shown). Indeed, they did not detect the toxin in culture filtrates from isolate 92-171R5, in which there was strong evidence to suggest that it is present at very low levels (Strelkov et al. 2002). Therefore, our failure

to detect Ptr ToxB from isolates D308 and 90-2 through Western blotting analysis is not conclusive evidence of its absence. The final explanation as to why no toxin activity was observed in isolates D308 and 90-2 could be related to its signal peptide. We were unable to obtain a sequence for the N-terminus of the signal peptide in isolates D308, 90-2, and 92-171R5 (Fig. 4.6 and Fig. 4.7). Although unlikely, there may be differences in that region that affect the ability of the fungal cells to transport Ptr ToxB out, making it unavailable as a tool in establishing compatibility between pathogen and host.

It is worth commenting on the observation that Ptr ToxB mRNA was detected in the ungerminated spores of *P. tritici-repentis* isolates (Fig. 4.4 and Fig. 4.5). Several other fungal species are known to produce dormant spores possessing stored mRNA that is translated upon germination. These include *Botryodiplodia theobromae* (Knight and Van Etten 1976), *Rhizopus stolonifer* (Van Etten and Freer 1978), *Allomyces macrogynus* (Smith and Burke 1979), *Mucor racemosus* (Linz and Orlowski 1982), *Neurospora crassa* (Plesofsky-Vig and Brambl 1985), and *Neurospora tetrasperma* (Plesofsky-Vig et al. 1992). Therefore, finding Ptr ToxB mRNA in the conidia of *P. tritici-repentis* is not surprising, given the importance of the toxin in establishing a compatible interaction between host and pathogen (at least in the case of isolates Alg 3-24, Alg-H2 and 92-171R5). The presence of Ptr ToxB mRNA in germinating fungal spores would allow for quick toxin synthesis, and thereby allow for faster infection of the wheat host.

The amino acid sequence and mass predicted here for Ptr ToxB from Alg 3-24 are slightly different from those reported by Strelkov et al. (1999) for the same isolate. Strelkov et al. (1999) reported a serine at residue 18, but in the present study we found that a cysteine residue is present at that position (Fig. 4.6 and Fig. 4.7). Indeed, a

cysteine was predicted at residue 18 not only in Alg 3-24, but also in all of the other isolates we studied (Fig. 4.6 and Fig. 4.7), as well as in isolate DW7 (Martinez et al. 2001). Thus, it is probable that the serine at residue 18 was an erroneous determination made through direct sequencing of the amino acids. The calculated mass of 6.54 kDa reported here for mature Ptr ToxB from isolates Alg 3-24, Alg-H2, 92-171R5 and D308, as well as by Martinez et al. (2001) for isolate DW7, is slightly lower than the 6.61 kDa value obtained by Strelkov et al. (1999) through mass spectrometry. It is possible that there are post-translational modifications to Ptr ToxB which alter its final mass, but that cannot be incorporated in calculations of mass from DNA sequences. Several potential sites for post-translational modification exist on the protein. It is clear that much remains to be elucidated regarding the nature of Ptr ToxB and its coding gene. Future studies could focus on obtaining genomic clones from all isolates possessing *ToxB*, determination of toxin tertiary structure and post-translational modifications, as well as advancing knowledge of its mechanism of action.

5.0 EFFECT OF *PYRENOPHORA TRITICI-REPENTIS* TOXINS ON STOMATAL APERTURE AND TRANSPIRATION OF WHEAT LEAVES

5.1 Abstract

Tan spot of wheat is caused by the fungus *Pyrenophora tritici-repentis*. This pathogen produces two host-specific toxins, Ptr ToxA and Ptr ToxB, which cause necrosis and chlorosis, respectively, on sensitive cultivars. In an effort to gain a better understanding of the processes involved in symptom development, the effect of the toxins on stomatal aperture and transpiration were investigated. Microscopic examination of epidermal strips from toxin-treated wheat leaves and controls suggested that neither toxin had an effect on stomatal function within the first 24 h post-infiltration. However, indirect measurement of transpiration rates revealed that treatment of sensitive tissue with Ptr ToxA caused a small decrease in transpiration relative to controls. Sensitive leaves treated with Ptr ToxB also lost less water than controls under light, but their transpiration rates did not decline under darkness, in contrast to controls. However, given the minor nature of the observed differences between toxin and control treatments, it seems unlikely that Ptr ToxA or Ptr ToxB have any direct effect on plant water or CO_2 exchange with the atmosphere.

5.2 Introduction

Tan spot is an important foliar disease of wheat caused by the fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoem.). This fungus produces at least two host-specific protein toxins, termed Ptr ToxA (formerly Ptr necrosis toxin, Ptr toxin or ToxA) (Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995; Zhang et al. 1997), and Ptr ToxB (formerly Ptr chlorosis toxin) (Orolaza et al. 1995; Strelkov et al. 1999). Ptr ToxA is responsible for development of necrosis on

sensitive cultivars, while Ptr ToxB causes chlorosis. Both toxins have been shown to be pathogenicity factors in the wheat-*P. tritici-repentis* interaction (Tomas and Bockus 1987; Ballance et al. 1989; Lamari and Bernier 1991; Orolaza et al. 1995; Ciuffetti et al. 1997).

Despite the importance of Ptr ToxA and Ptr ToxB in the development of tan spot, little is known regarding their modes of action. Inhibitors of transcription and translation were found to reduce the level of electrolyte leakage induced by Ptr ToxA, which led Kwon et al. (1998) to suggest that action by this toxin requires active host-processes. Ptr ToxB was shown to cause a light-dependent degradation of chlorophyll, likely as a consequence of the formation of active oxygen (AO) species, resulting from a direct or indirect inhibition of photosynthesis (Strelkov et al., 1998). However, little else is known regarding the underlying mechanisms leading to symptom development in response to these toxins. Therefore, the present study was undertaken to examine the effects of Ptr ToxA and Ptr ToxB on stomatal aperture and transpiration, to further characterize the changes they cause in sensitive tissue.

5.3 Materials and Methods

Plant materials. Wheat cvs. Glenlea (sensitive to Ptr ToxA), Katepwa (sensitive to Ptr ToxA and Ptr ToxB) and Erik (insensitive to both toxins) were used throughout this study. Unless otherwise stated, seedlings were grown in plastic pots, 12 cm diameter, filled with a 1:1 soil/peat mix. Plants were seeded at a rate of 5 to 6 seeds per pot and maintained in a growth room at 22/18^{0}C (day/night) with a 16 h photoperiod, at a light intensity of 250 μmoles/m^2/s and 49% relative humidity. The plants were watered and fertilized as required.

Toxins and infiltration of tissues. The second leaves of seedlings at the 2-3 leaf stage were infiltrated with approximately 100 μL of either water, 10 μg/mL Ptr ToxA, or 7.9 μg/mL Ptr ToxB, using a Hagborg device (Hagborg 1970). The toxins were purified according to

previously published methods (Ballance et al. 1989; Strelkov et al. 1999). The infiltrated zones extended approximately 7 cm along the length of the leaf, beginning approximately 2 cm from the leaf tip.

Microscopic examination of stomata. Epidermal strips from wheat leaves were prepared as described in Weyers and Meidner (1990), at 3, 6, 12, 24, 36 and 48 h after infiltration with water, Ptr ToxA, or Ptr ToxB. The strips were stained for starch with potassium iodide, and examined under a compound microscope (Standard 18, Carl Zeiss Canada Ltd., Toronto, Ontario).

Transpiration bioassay. This assay was based on that described by Biddington and Thomas (1978). Second leaves of wheat seedlings at the 2-3 leaf stage, treated with either distilled water or one of the toxins, were cut 10 cm from the leaf tip immediately after infiltration. Glass beakers (10 mL volume), each containing 5 mL distilled water, were covered with aluminum foil and weighed. One 10 cm leaf segment was stood in each beaker by inserting it through a slit made in the aluminum foil, and the beakers were re-weighed. Transpiration was measured by recording the weight lost from the beakers and the leaves over a time-course. Changes in leaf weight were calculated by weighing the beakers without leaves at the end of the experiment. Evaporative water loss was estimated by measuring weight lost in beakers covered in aluminum foil but containing no leaves. Transpiration was expressed as the weight of water lost per beaker. Beakers were kept in the same growth room and under the same conditions as the seedlings, as described above. Experiments were repeated three times, and treatments were replicated three times in each run. Results presented are from a typical run.

5.4 Results

Microscopic examination of stomata. Necrosis symptoms in toxin-sensitive seedlings were clearly visible within 24 h post-infiltration with Ptr ToxA. After this time, it became very difficult to obtain good epidermal strips. In Ptr ToxB-sensitive seedlings, chlorosis began to appear approximately 48 h post-infiltration with Ptr ToxB. Epidermal strips could be obtained up to this time, but with increasing difficulty. Thus, only epidermal strips obtained within 24 h of infiltration with either toxin were examined. During the initial 24 h post-infiltration period, no significant differences in stomatal aperture were observed between sensitive tissues infiltrated with Ptr ToxA or Ptr ToxB, insensitive tissues infiltrated with either toxin, or sensitive or insensitive tissues infiltrated with water. At 12 h post-infiltration, stomata from 'Glenlea' seedlings (Ptr ToxA-sensitive), which were heavily watered and infiltrated with Ptr ToxA, were open (Fig. 5.1A), while stomata from 'Glenlea' seedlings infiltrated with Ptr ToxA but kept in very dry soil were closed (Fig. 5.1B). Similarly, stomata from 'Katepwa' seedlings (Ptr ToxB-sensitive), which were heavily watered and infiltrated with Ptr ToxB, were open, while those from 'Katepwa' seedlings infiltrated with Ptr ToxB but kept in very dry soil were closed (results not shown). All controls showed the same pattern - open stomates in heavily watered seedlings, and closed stomates in seedlings growing in very dry soil.

Transpiration bioassay. Changes in leaf weight accounted for a negligible fraction of the total weight loss from each beaker (never more than 1.4% of the total). Weight loss from

Figure 5.1. Effect of Ptr ToxA on stomatal aperture. (A) and (B) Views of epidermal strips (magnified 400X) from wheat leaves, stained with potassium iodide. (A) Open stomate from leaf of a heavily watered 'Glenlea' seedling infiltrated with Ptr ToxA. (B) Closed stomate from leaf of a 'Glenlea' seedling infiltrated with Ptr ToxA and growing in very dry soil. The same trends were observed with stomates of leaves from controls or infiltrated with Ptr ToxB; stomates were open in seedlings growing in wet soil, but were closed in seedlings growing in dry soil.

beakers containing no leaves was minimal (eg. 0.0014 g over 12 h) and constant (Fig. 5.2 and Fig. 5.3). Given the earlier onset of necrosis as compared to chlorosis, transpiration time-courses for leaves treated with Ptr ToxA were shorter than those for leaves treated with Ptr ToxB (12 h vs. 24 h, respectively). The transpiration profiles for 'Glenlea' leaves infiltrated with Ptr ToxA resembled those for water-infiltrated 'Glenlea' leaves (Fig. 5.2). However, the total water loss over time at each time point was higher for the Glenlea + water treatment than for the Glenlea + Ptr ToxA treatment (Fig. 5.2). Rates of water loss declined for both treatments over the time course. From 0 to 4 h post-infiltration, water loss from the Glenlea + water and Glenlea + Ptr ToxA treatments averaged 52.5 mg/h and 35.4 mg/h, respectively. From 4 to 8 h, this rate declined to 45.9 mg/h and 33.0 mg/h for the Glenlea + water and Glenlea + Ptr ToxA treatments, respectively. The rate of water loss declined further over the 8 to 12 h post-infiltration period, to 36.7 mg/h for the Glenlea + water treatment and 24.9 mg/h for the Glenlea + Ptr ToxA treatment. For both treatments, the rate of water loss for the 8 to 12 h period was approximately 70% of the rate for the 0 to 4 h period. The growth chamber was under light during the entire length of the assay.

'Katepwa' leaves infiltrated with Ptr ToxB transpired slightly less than controls infiltrated with water over the length of the time course (Fig. 5.3). However, the transpiration profiles were not as similar as they were with the Glenlea + Ptr ToxA vs. Glenlea + water treatments. The rate of water loss from the Katepwa + water treatment remained stable at 0 to 6 and 6 to 12 h, ranging from 32.4 mg/h to 34.5 mg/h. However, after 12 h, when the growth chamber lights went off, the rate dropped sharply to 18.9 mg/h. In contrast, the rate of water loss from the Katepwa + toxin treatment remained quite stable

Figure 5.2. Effect of Ptr ToxA on transpiration of excised wheat cv. Glenlea leaves. Second leaves of wheat seedlings at the 2-3 leaf stage were cut 10 cm from leaf tip, after infiltration with Ptr ToxA or water, and placed in 10 mL beakers sealed with aluminum foil and filled with 5 mL water. Weight loss from the beakers was measured over time for leaves treated with Ptr ToxA or water. Beakers containing 5 mL water and covered with aluminum foil, but containing no leaves, were included to estimate water loss due to evaporation.

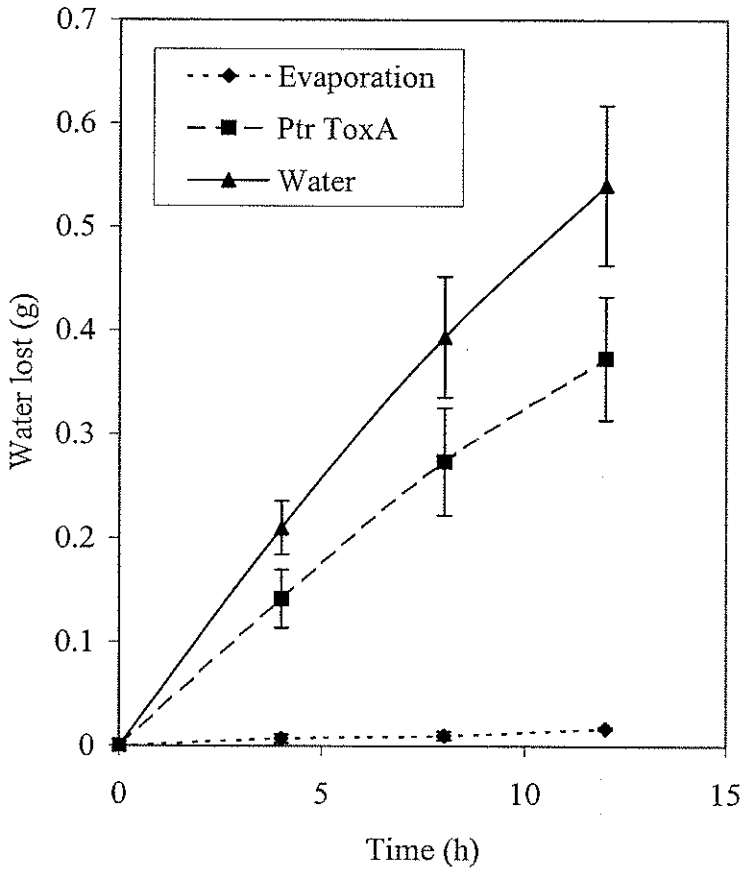
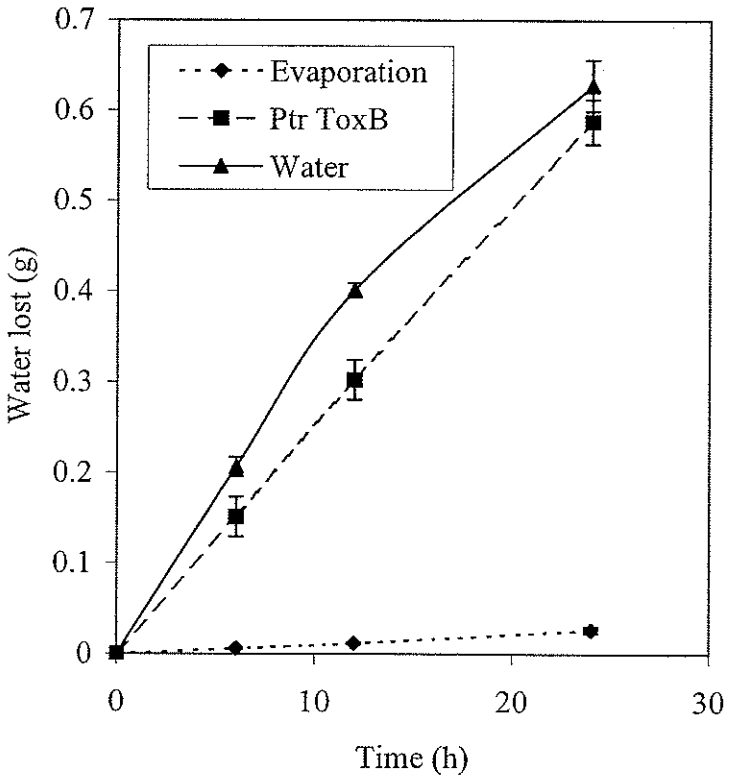

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Water lost (g)
Evaporation
Ptr ToxA
Water
0
5
10
15
Time (h)

Figure 5.3. Effect of Ptr ToxB on transpiration of excised wheat cv. Katepwa leaves. Second leaves of wheat seedlings at the 2-3 leaf stage were cut 10 cm from leaf tip, after infiltration with Ptr ToxB or water, and placed in 10 mL beakers sealed with aluminum foil and filled with 5 mL water. Weight loss from the beakers was measured over time for leaves treated with Ptr ToxB or water. Beakers containing 5 mL water and covered with aluminum foil, but containing no leaves, were included to estimate water loss due to evaporation.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Water lost (g)
Evaporation
Ptr ToxB
Water
0
10
20
30
Time (h)

over the entire length of the assay. For this treatment, water losses averaged 25.3 mg/h, 25.2 mg/h and 23.8 mg/h from 0 to 6, 6 to 12 and 12 to 24 h, respectively.

5.5 Discussion

Several toxins have been reported to have an effect on stomatal aperture, and this effect could at least partially account for the symptoms they produce. For example, fusicoccin, produced by *Fusicoccum amygdali*, induces stomatal opening, leading to uncontrolled transpiration and eventually to wilt (Heiser et al., 1998). Tentoxin, in addition to binding to the α,β-subunit of chloroplast ATPase and inhibiting photophosphorylation, may also cause a rapid closure of broad bean stomates (Durbin et al. 1973), and counteracts fusicoccin-induced stomatal opening in the dark (Bocher and Novacky 1981). The possibility that the Ptr toxins also have an effect on stomatal aperture and transpiration was investigated, given the nature of the symptoms they produce. Necrosis caused by Ptr ToxA is typified by severe desiccation of the affected tissue. It was hypothesized that this could be indicative of altered water relations, perhaps including induced stomatal opening. Similarly, it was also postulated that chlorosis and the formation of AO species in response to Ptr ToxB (Strelkov et al. 1998) could result from an inhibition of the Calvin cycle as a consequence of stomatal closure and reduced entry of CO_2 into the leaves.

Based on the microscopic examination of stomata, it would appear that neither Ptr ToxA nor Ptr ToxB have a significant effect on stomatal function, at least not within 24 h post-infiltration with either of the toxins (Fig. 5.1). Stomates in sensitive, toxin-treated tissues behaved in the same manner as controls. They were open in heavily watered seedlings, but were closed in seedlings growing in very dry soil (Fig. 5.1). This is what would have been expected in normally functioning stomates; as seedlings become water

stressed, stomata close to conserve water. However, the microscopic study was purely qualitative in nature. Epidermal strips were examined to determine whether or not stomata were open, but no measurements were made to quantify average stomatal aperture among the different treatments.

Indeed, when transpiration rates were measured indirectly by monitoring water loss from vials, it was found that sensitive leaves treated with Ptr ToxA transpired less than water-infiltrated controls (Fig. 5.2). Similarly, sensitive leaves treated with Ptr ToxB lost less water than controls in the first 12 h of that assay (Fig. 5.3). Although the differences in transpiration were relatively small, they suggest that average stomatal aperture may have been less in toxin-treated sensitive tissue than in controls. However, after 12 h in the Ptr ToxB assay, despite the onset of darkness in the growth chamber, transpiration rates did not decline in the Katepwa + toxin treatment, but instead remained constant (Fig. 5.3). Such a decline would have been expected, and was in fact observed in the Katepwa + water control, since the need for CO_2 for photosynthetic carbon reduction would be reduced. This observation suggests that the ability of 'Katepwa' leaves to respond to changes in their environment may have been impaired. Thus, it appears that alterations in plant physiological processes commenced relatively early after treatment of sensitive tissue with Ptr ToxA or Ptr ToxB. However, given the small magnitude of the changes and their somewhat general nature, they were likely secondary consequences of other metabolic and biochemical changes beginning to occur in affected tissues, and probably reflected leaf stress.

If chlorosis in response to Ptr ToxB developed as a result of CO_2 starvation, as has been suggested to occur with tentoxin (Durbin et al. 1973; Bocher and Novacky 1981), then stomates would have been closed under all conditions, even those in which they would

normally be open. Furthermore, a sharp decline in the transpiration rates of affected leaves would have been expected as the stomates began to close. However, neither of these effects was observed (Fig. 5.3). If Ptr ToxA caused necrosis/dessication by a mechanism involving abnormal stomatal opening, in a manner analogous to fusicoccin, then stomates would have been open under all conditions, and transpiration rates would have increased substantially, yet this is not what was found (Fig. 5.1 and Fig. 5.2). Therefore, despite the observed differences in water loss from toxin-treated tissues and controls (Fig. 5.2 and Fig. 5.3), Ptr ToxA and Ptr ToxB appear to have little significant effect on stomatal aperture and transpiration. Thus, the symptoms they cause in sensitive tissues appear to arise via mechanisms that do not directly involve water or CO_2 exchange with the atmosphere.

6.0 PTR TOXB INDUCED CHANGES IN FREE AMINO ACID POOLS OF WHEAT SEEDLING LEAVES

6.1 Abstract

The fungus *Pyrenophora tritici-repentis* causes tan spot, an important foliar disease of wheat. Previous studies demonstrated that Ptr ToxB, a host-specific toxin produced by the pathogen, induces an AO-mediated photo-oxidation of chlorophyll in sensitive tissue, likely as a result of the inhibition of photosynthesis. In the present study, we investigated the effect of Ptr ToxB on the level of total free amino acids in the leaves of toxin-sensitive wheat seedlings and controls, as well as its effect on the relative amounts of individual amino acids. The toxin was found to induce large accumulations of total free amino acids in toxin-sensitive leaves, commencing at 48 h post-infiltration. These accumulations were probably due to increased protein degradation and a decreased utilization of amino acids in protein synthesis. The relative pool size of Glu + Gln was found to increase, beginning at 48 h, as did the levels of the branched chain amino acids Leu, Val and Ile. The level of Pro also increased greatly in toxin-treated sensitive tissue, but beginning at 72 h, likely as a result of leaf stress. In contrast, amounts of Ser, Gly and Ala decreased substantially starting at 48 h. We postulate that the declines in Gly and Ser may be due to the deficiency in Ala, resulting in depletion of amino donors for the conversion of glyoxylate to Gly, which ultimately leads to an inhibition of photosynthesis.

6.2 Introduction

Tan spot is an important foliar disease of wheat caused by the fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Dreschslera tritici-repentis*

(Died.) Shoem.). The fungus selectively induces tan necrosis and/or chlorosis on its wheat host. Isolates of *P. tritici-repentis* are classified into races based on their ability to cause these symptoms on a wheat differential set (Lamari et al. 1995). To date, six races have been identified (Lamari et al. 1995; Strelkov et al. 2002). The ability of the fungus to infect its host and induce symptoms appears to be directly related to its ability to produce one or more of at least three known host-specific toxins (Lamari and Bernier 1989; Orolaza et al. 1995; Effertz et al. 2002; Strelkov et al. 2002).

The necrosis symptom that develops in response to infection by races 1 and 2 of *P. tritici-repentis* is caused by the action of a host-specific toxin termed Ptr ToxA (formerly Ptr necrosis toxin, Ptr toxin, ToxA) (Lamari and Bernier 1989). Action by this toxin seems to require active host-processes, including transcription and translation (Kwon et al. 1998). However, the exact nature of the wheat-Ptr ToxA interaction remains to be elucidated. Chlorosis in response to infection by races 5 and 6 of the pathogen is due to their production of another host-specific toxin, termed Ptr ToxB (formerly Ptr chlorosis toxin) (Orolaza et al. 1995; Strelkov et al. 2002). Induction of chlorosis by Ptr ToxB was observed to be strictly light dependent and independent of tissue age, suggesting that chlorophyll is being photooxidized (Strelkov et al. 1998). Furthermore, the active oxygen (AO) scavenger p-benzoquinone was found to prevent development of the chlorosis symptom, indicating that AO species are involved in the photobleaching of chlorophyll (Strelkov et al. 1998). Results also indicated that toxin-induced chlorosis was not due to a carotenoid deficiency (Strelkov et al. 1998). These observations led Strelkov et al. (1998) to conclude that Ptr ToxB directly or indirectly inhibits photosynthesis, leading to the photodestruction of chlorophyll. Despite this basic

knowledge regarding the mode of action of the toxin, much remains unknown. The effect of Ptr ToxB on the free pool sizes of total foliar amino acids, as well as its effect on the relative amounts of individual amino acids, was examined in order to better characterize the host response to toxin-treatment.

6.3 Materials and Methods

Plant materials. Wheat cultivars Katepwa (sensitive to Ptr ToxB) and Glenlea (insensitive to Ptr ToxB) were grown in plastic pots (12cm diameter) filled with a 1:1 soil:peat mix. Plants were seeded at a rate of 5 to 6 seeds per pot, and kept in a growth room at 22^0C/18^0C (day/night) with a 16 h photoperiod, at a light intensity of approximately 250 μmoles/m^2/s. Seedlings were watered and fertilized as necessary.

Treatment with Ptr ToxB. Ptr ToxB was purified as previously described (Strelkov et al. 1999) from *P. tritici-repentis* race 5 isolate Alg 3-24. Toxin sensitive and insensitive seedlings were infiltrated with either water or toxin (at 7.9 μg/mL) using a Hagborg device (Hagborg 1970). All experiments were repeated three times. For determination of total amino acids, treatments were replicated 3 times in each run of the experiment, and the results presented are from a typical run. For determination of amino acid composition, one sample of each treatment per experiment was sent for amino acid analysis, and the results presented are averages from the three repetitions of the experiment.

Extraction of free amino acids. Total free amino acids were extracted from infiltrated leaf tissue according to the method of Bieleski and Turner (1966). Leaf segments, approximately 100mg fresh weight, were frozen and ground in liquid nitrogen. They were then extracted two times in methanol:chloroform:water (MCW, 12:5:3, v:v:v) and

once in 80% (v:v) ethanol. The aqueous phase of the MCW extract was combined with the ethanol extract, and the combined extract dried in a rotovapor at 40^0C.

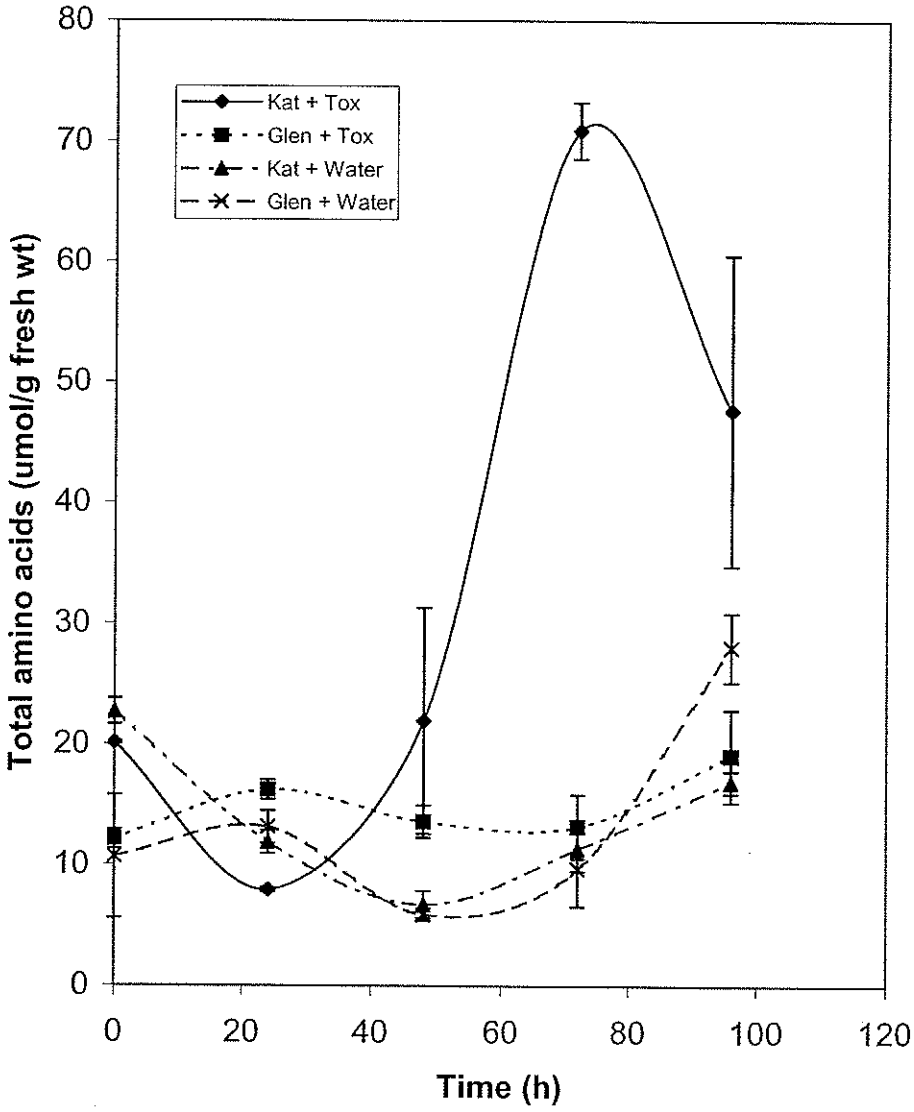
Determination of total amino acids. Dried amino acid extracts were dissolved in distilled water and free amino acid content was determined with ninhydrin, as described by Yemm and Cocking (1955), using a leucine standard curve. Twenty microliters of each sample were retained and used to correct for ammonia (Weatherburn 1967).

Amino acid composition analysis. Dried amino acid extracts were dissolved in distilled water and amino acids were further purified by passage through Microcon-SCX Adsorptive Microconcentrators (Millipore, Bedford, MA), as per manufacturer's instructions. Amino acids were eluted from the microconcentrators using 6N HCl/Methanol, and dried down as above. Samples were re-dissolved in distilled water, and sent for amino acid analysis to the Protein Microsequencing Laboratory, University of Victoria, Victoria, BC. Amino acid composition was determined using an Applied Biosystems (Foster City, CA) model 420A amino acid derivatizer-analyzer.

6.4 Results and Discussion

There were no differences in levels of total foliar amino acids between any of the treatments until approximately 48 h post-infiltration with Ptr ToxB or water (Fig. 6.1). It was at this time that the level of free amino acids began to rise in the Katepwa + toxin treatment (Fig. 6.1). This increase continued until approximately 72 h post-infiltration, when the level of free amino acids in toxin-treated 'Katepwa' leaves was approximately seven-fold greater than in the other treatments (Fig. 6.1). After 72 h, the free amino acid content in the Katepwa + toxin treatment begun to decline, although at 96 h it remained two to three-fold higher than in the other treatments (Fig. 6.1). Previously,

Figure 6.1. Total free amino acids in leaves of wheat seedlings over time after treatment with Ptr ToxB or water. Total free amino acids were determined at various times after infiltration of toxin-sensitive 'Katepwa' (Kat) and toxin-insensitive 'Glenlea' (Glen) leaves with either distilled water or toxin (7.9μg/mL). Error bars indicate the standard deviation.


Kat + Tox
Glen + Tox
Kat + Water
Glen + Water
Total amino acids (umol/g fresh wt)
Time (h)
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100
120

the onset of Ptr ToxB-induced chlorosis was found to occur at approximately 48 h post-infiltration (Strelkov et al. 1998). It is interesting that the increase in foliar amino acids reported here also commenced at 48 h. This suggests that the accumulation of free amino acids, at least at later time points, may be a secondary consequence of the widespread, AO-mediated damage that also causes chlorosis. Amino acids probably accumulated as a result of increased protein degradation in affected tissue, as well as their decreased utilization in protein synthesis. After 72 h, as cellular damage became progressively more severe, the amino acids themselves probably begun to be degraded, explaining their decline after this time in the Katepwa + toxin treatment.

There were no significant differences between the treatments with respect to the relative amounts of individual amino acids at 0 and 24 h post-infiltration with Ptr ToxB or water (results not shown). However, at 48 h post-infiltration, the relative levels of Ser, Gly and Ala had declined in the Katepwa + toxin treatment, when compared to the other treatments (Fig. 6.2). In contrast, the relative amounts of Val, Ile and Leu were significantly higher in this treatment (Fig. 6.2). The level of Glu + Gln (Glx), although still within error, was nevertheless also higher in the Katepwa + toxin treatment (Fig. 6.2). Relative amounts of the other amino acids were consistent throughout all treatments. At 72 h post-infiltration, the decline in the relative quantities of Ser, Gly and Ala continued in the Katepwa + toxin treatment (Fig. 6.3). By this point, they were less than half the levels in the control treatments (Fig. 6.3). Relative amounts of the branched-chain amino acids (Val, Ile and Leu), remained greater in toxin-treated 'Katepwa' leaves than in the controls (Fig. 6.3). Likewise, the relative level of Glu + Gln was also greater, the difference becoming significant by this time (Fig. 6.3). Proline

Figure 6.2. Relative foliar amino acid composition (mol %) of wheat seedlings 48 h after treatment with Ptr ToxB or water. Only the values for toxin-sensitive 'Katepwa' (Kat) leaves, infiltrated with either distilled water or toxin (7.9μg/mL), are shown; the values for toxin-insensitive 'Glenlea' leaves, infiltrated with distilled water or toxin, were very similar to those of the Kat + Water treatment. Asx = Asp + Asn, Glx = Glu + Gln. Error bars indicate the standard deviation.

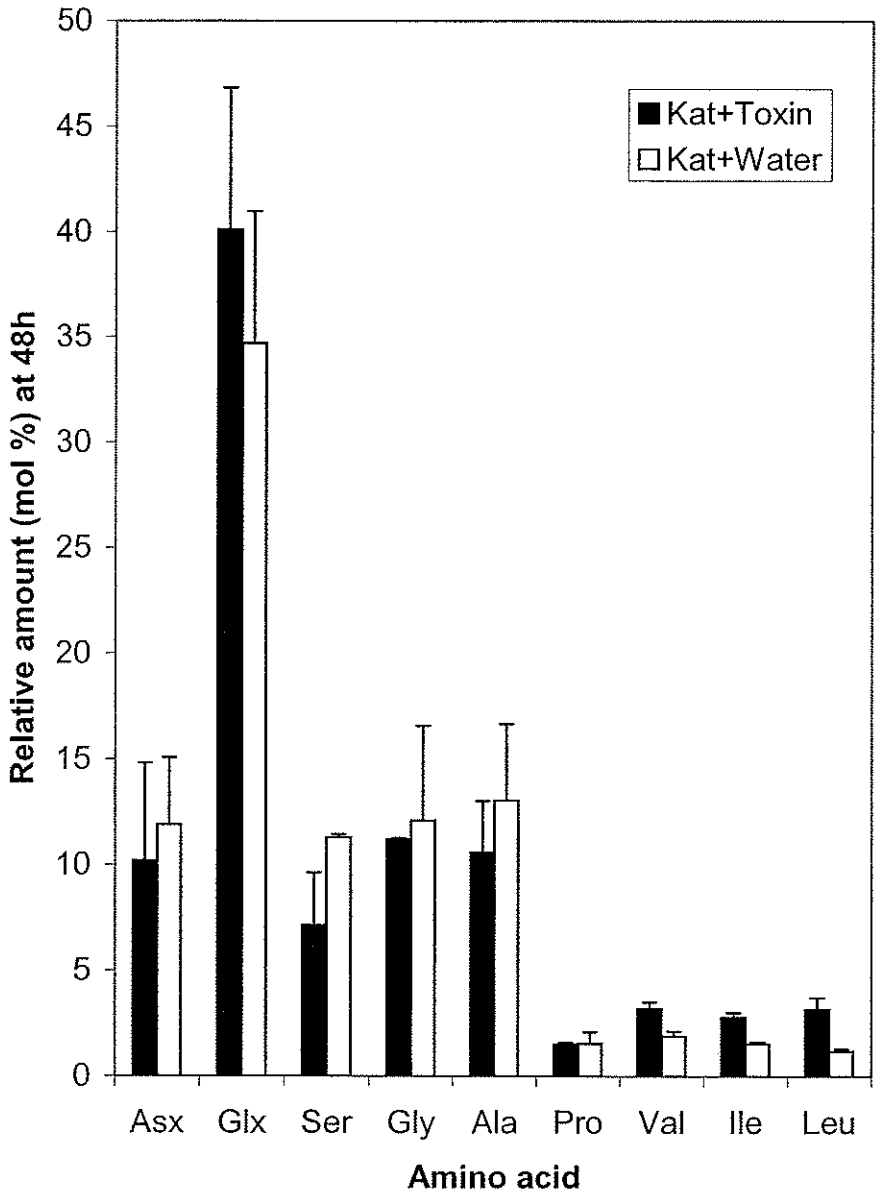
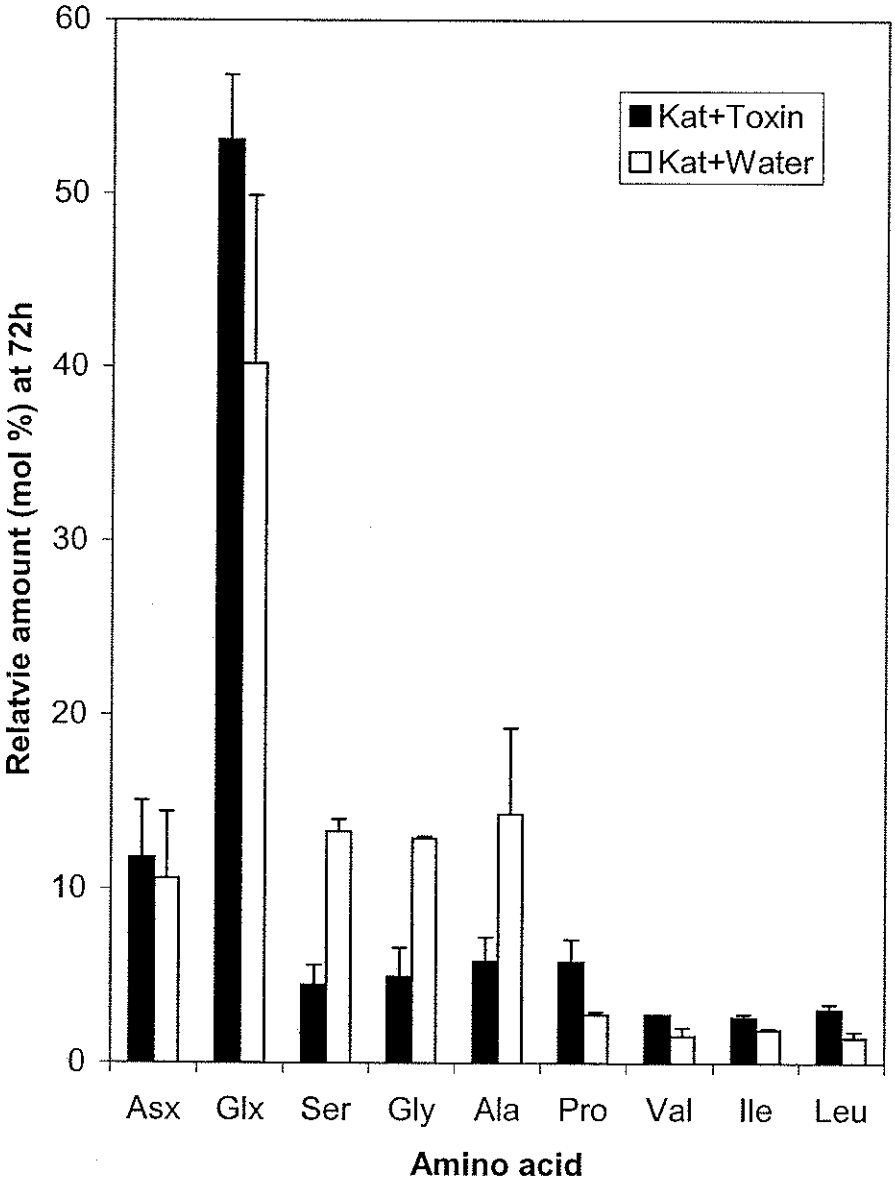

50
45
40
35
30
25
20
15
10
5
0
Relative amount (mol %) at 48h
Kat+Toxin
Kat+Water
Asx
Glx
Ser
Gly
Ala
Pro
Val
Ile
Leu
Amino acid

Figure 6.3. Relative foliar amino acid composition (mol %) of wheat seedlings 72 h after treatment with Ptr ToxB or water. Only the values for toxin-sensitive 'Katepwa' (Kat) leaves, infiltrated with either distilled water or toxin (7.9μg/mL), are shown; the values for toxin-insensitive 'Glenlea' leaves, infiltrated with distilled water or toxin, were very similar to those of the Kat + Water treatment. Asx = Asp + Asn, Glx = Glu + Gln. Error bars indicate the standard deviation.

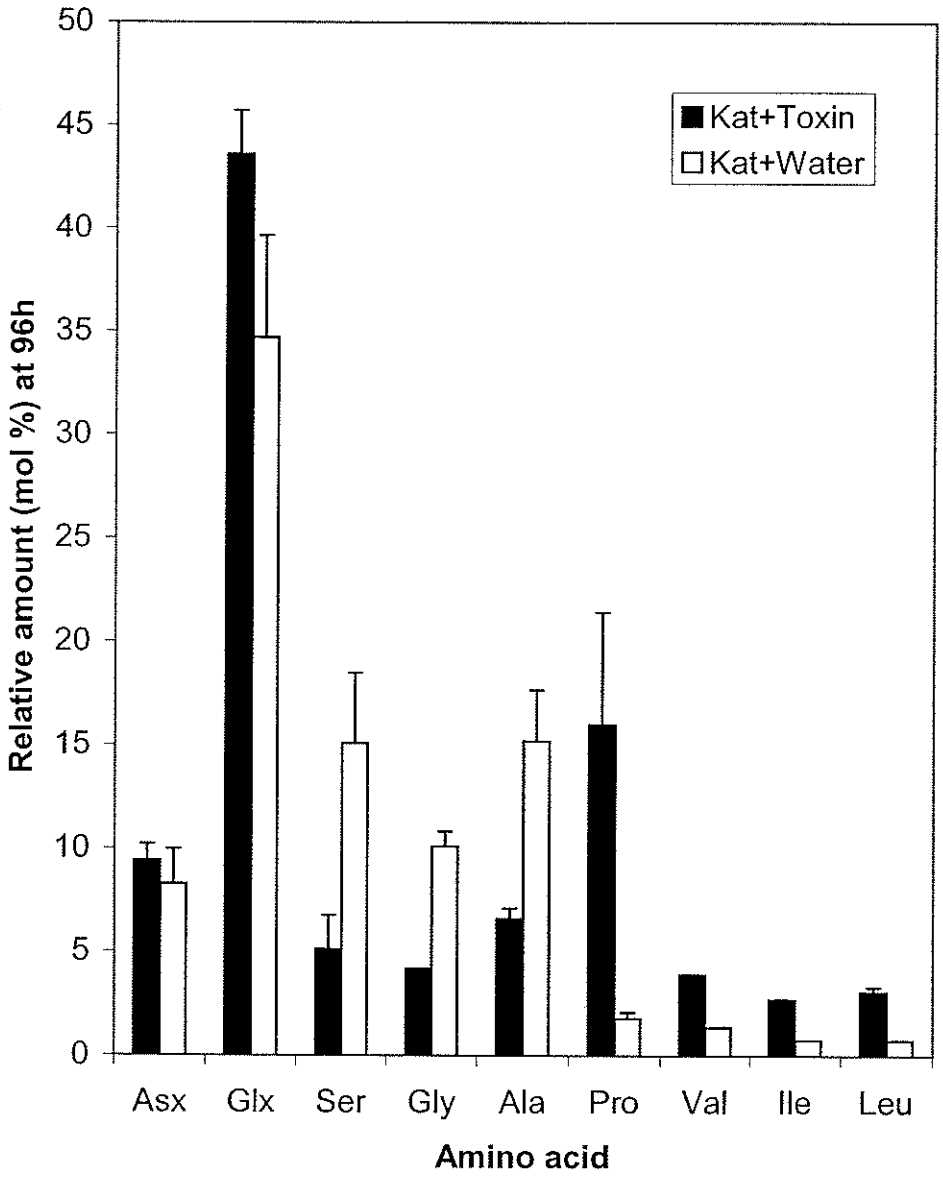

60
50
40
30
20
10
0
Relatvie amount (mol %) at 72h
Kat+Toxin
Kat+Water
Asx
Glx
Ser
Gly
Ala
Pro
Val
Ile
Leu
Amino acid

levels, which had been similar among all treatments at earlier time points, were approximately twice as high in the Katepwa + toxin treatment at 72 h (Fig. 6.3). The same general trends were observed at 96 h post-infiltration. At this time, the relative amounts of Ser, Gly and Ala in the Katepwa + toxin treatment ranged from a third to less than half the amounts in the other treatments (Fig. 6.4). Relative levels of Val, Ile and Leu in toxin-treated 'Katepwa' were more than twice that found in the other treatments (Fig. 6.4). The quantity of Glu + Gln also remained significantly higher in the Katepwa + toxin treatment (Fig. 6.4). The relative level of Pro continued to increase in the toxin-treated 'Katepwa' leaves, with approximately eight times as much Pro in this treatment compared with the others (Fig. 6.4). No clear trends were discernable over time in the relative quantities of the other amino acids. At 72 and 96 h post-infiltration, the level of Asp + Asn (Asx) was highest in the Katepwa + toxin treatment, but it was still well within error (Fig. 6.3 and Fig. 6.4).

As was the case with the onset of chlorosis (Strelkov et al. 1998) and the rise in total free amino acids in toxin-treated 'Katepwa' leaves (Fig. 6.1), changes in the relative composition of free foliar amino acids in response to Ptr ToxB-treatment were not detected until 48 h post-infiltration. Nevertheless, at this and later times, numerous changes in amino acid composition were observed in the Katepwa + toxin treatment. These changes may reflect a general deregulation of amino acid biosynthesis (Höfgen et al. 1995). More significantly, however, they may also reflect specific changes induced by Ptr ToxB in amino acid and other metabolic pathways.

Herbicides such as phosphinothricin (Manderscheid and Wild 1986) and the host-nonspecific toxin tabtoxin (Turner 1981) cause their associated symptoms through the

Figure 6.4. Relative foliar amino acid composition (mol %) of wheat seedlings 96 h after treatment with Ptr ToxB or water. Only the values for toxin-sensitive 'Katepwa' (Kat) leaves, infiltrated with either distilled water or toxin (7.9μg/mL), are shown; the values for toxin-insensitive 'Glenlea' leaves, infiltrated with distilled water or toxin, were very similar to those of the Kat + Water treatment. Asx = Asp + Asn, Glx = Glu + Gln. Error bars indicate the standard deviation.


50
45
40
35
30
25
20
15
10
5
0
Relative amount (mol %) at 96h
Kat+Toxin
Kat+Water
Asx
Glx
Ser
Gly
Ala
Pro
Val
Ile
Leu
Amino acid

inhibition of glutamine synthetase (GS), the main enzyme of ammonium assimilation in higher plants (Woodall et al. 1996). In the case of tabtoxin, Turner (1988) suggested that the inactivation of GS results in a lesion in the photorespiratory nitrogen cycle, leading to an inhibition of photosynthesis either through a reduced return of photorespiratory cycle intermediates to the Calvin cycle, or the uncoupling of photophosphorylation by ammonia. Similarly, declines in photosynthetic CO_2 fixation caused by phosphinothricin appear to result from an inhibition of photorespiration (Wendler et al. 1990). However, the fact that Glu + Gln and Asp + Asn levels did not decrease in the Katepwa + toxin treatment suggests that Ptr ToxB does not affect GS activity, at least initially. Indeed, the relative amount of Glu + Gln is highest in this treatment (Fig. 6.2, Fig. 6.3 and Fig. 6.4). Accumulation of Gln has been reported to occur when the N-supply exceeds its demand in protein synthesis (Millard 1988), and therefore the observed increase in Glu + Gln may reflect disruptions in protein synthesis and metabolism induced by Ptr ToxB.

Nevertheless, the relative amounts of Ser and Gly in toxin-treated 'Katepwa' leaves were much lower than in any other treatment (Fig. 6.2, Fig. 6.3 and Fig. 6.4). This is simlar to what has been found in response to treatment of C3 plants with phosphinothricin (Wendler et al. 1990; Shelp et al. 1992). The inhibition of GS by phosphinothricin results in a decrease in Glu as well as Gln (Sauer et al. 1987), and as a result the transamination of glyoxylate to glycine in photorespiration cannot occur (Wallsgrove et al. 1987), since Glu is an amino donor in this reaction. In the case of Ptr ToxB, there is no lack of Glu + Gln (Fig. 6.2, Fig. 6.3 and Fig. 6.4), and therefore Glu is still available as an amino donor. However, the transaminase enzyme capable of converting glyoxylate to Gly, glutamate (alanine)-glyoxylate aminotransferase, also uses

Ala as an amino donor. Although the affinity of this enzyme is identical for both Glu and Ala (Nakamura and Tolbert 1983), it has been estimated that there is a three times higher rate of nitrogen flux from Ala to Gly than from Glu to Gly (Betsche 1983; Betsche and Eising 1986). Interestingly, we found that the relative amount of Ala in the Katepwa + toxin treatment was lower than in any other treatment (Fig. 6.2, Fig. 6.3 and Fig. 6.4). This raises the possibility that an Ala deficiency results in a depletion of amino donors for the conversion of glyoxylate to Gly, resulting in a lesion in the photorespiratory nitrogen cycle. This could in turn lead to the inhibition of photosynthesis either through the uncoupling of photophosphorylation by ammonia, or through the reduced return of photorespiratory cycle intermediates to the Calvin cycle. Unfortunately, although ammonia levels were monitored to correct determinations of total free amino acids, no thorough, quantitative investigation into its accumulation was undertaken here. Thus, the hypothesis presented above is highly speculative. Nevertheless, we also observed large increases in the levels of branched chain amino acids (Fig. 6.2, Fig. 6.3 and Fig. 6.4), just as was observed in leaf tissue treated with phosphinothricin (Wendler et al. 1990; Shelp et al. 1992).

The relative amount of free Pro also increased in the Katepwa + toxin treatment when compared to the other treatments (Fig. 6.3 and Fig. 6.4). However, the change in the level of this amino acid occurred later than changes in the free pool sizes of other amino acids, since it was first noticeable at 72 h rather than 48 h. However, by 96 h, the increases in Pro were among the most dramatic observed (Fig. 6.4). Accumulation of free Pro has been reported in different tissues of plants grown under stress (Singh et al. 1973; Navari-Izzo et al. 1990; Girousse et al. 1996), including growing, mature and

detached leaves (Stewart 1972; Lawlor and Fock 1977; Riazi et al. 1985). Indeed, Pro accumulation has been used as an indicator of moisture or light stress (Joyce et al. 1984; McLaughlin et al. 1994). Thus, the increases in Pro that we observed probably reflect the fact that toxin-treated sensitive tissue was under stress. It is therefore not surprising that Pro levels did not begin to increase until well after the levels of other amino acids had begun to fluctuate, as Pro accumulated as a consequence of profound physiological disturbances in affected tissue.

The exact basis for biological action by Ptr ToxB remains a mystery. Previously, it was shown that the toxin induces AO-mediated photo-oxidation of chlorophyll, likely as a result of an inhibition of photosynthesis (Strelkov et al. 1998). The present investigation provided indirect evidence that a lesion in the photorespiratory nitrogen cycle may lead to this inhibition, and ultimately to the development of chlorosis. However, this hypothesis is highly speculative. Many questions need to be answered, including what causes the initial decline in Ala. Future studies should examine the effect of Ptr ToxB on ammonia accumulation in sensitive tissue, as well as its effect on various enzymes involved in amino acid metabolism, particularly alanine dehydrogenase and alanine aminotransferase.

7.0 GENERAL DISCUSSION

7.1 The Ptr Toxins as Pathogenicity Factors

Previous studies have shown that the Ptr toxins function as pathogenicity factors (Tomás and Bockus 1987; Lamari and Bernier 1991; Orolaza et al. 1995; Ciuffetti et al. 1997). This fact was most clearly illustrated by the transformation of a tox$^-$ isolate of *P. tritici-repentis* with a vector containing a genomic copy of the *ToxA* gene (Ciuffetti et al. 1997). Expression of this gene resulted in the conversion of the avirulent isolate into a pathogenic isolate capable of causing disease (Ciuffetti et al. 1997). Thus, the ability to produce Ptr ToxA was the only trait the avirulent isolate lacked in order to overcome resistance and cause disease (Ciuffetti et al. 1997).

If the Ptr toxins are pathogenicity factors, then the ability of different isolates to produce particular toxins should have a direct effect on the range of wheat lines or cultivars that they can infect. This is precisely what is observed. Isolates from race 1 produce both Ptr ToxA and Ptr ToxC, and therefore their virulence pattern combines those of isolates from race 2, which produce only Ptr ToxA, and race 3, which produce only Ptr ToxC (Lamari and Bernier 1989c; Effertz et al. 2002). Similarly, race 6 isolates produce both Ptr ToxB and Ptr ToxC, and their virulence pattern combines those of isolates belonging to race 5, which produce only Ptr ToxB, and race 3 (Orolaza et al. 1995; Effertz et al. 2002; Section 3.0 of thesis). Race 4 isolates produce no active toxins and are therefore avirulent. The presence of Ptr ToxB mRNA in the conidia of *P. tritici-repentis* may reflect the importance of the Ptr toxins in establishing a compatible interaction between host and pathogen (Section 4.0). If Ptr ToxB is needed by the fungus to overcome basic host resistance, then it is not surprising that dormant spores would

possess stored Ptr ToxB mRNA that is translated upon germination. This would allow for more rapid toxin synthesis and earlier infection of the host.

The ability of *P. tritici-repentis* isolates to produce particular host-specific toxins could have a profound effect on the evolution of this pathogen. The race differentiation observed may ultimately lead to the development of different formae speciales and eventually species. Speciation requires barriers to gene flow such as ecological and genetic isolation between populations (Hansen 1987). The host specificity provided by the Ptr toxins could create ecological barriers between races, by allowing them to become adapted to different niches (ie. different hosts). This effect could be enhanced by the use of monoculture, which also provides opportunities for the speciation of pathogenic fungi, since it favours host-specific pathogenicity by supplying hosts for specialized fungal pathogens (Hansen 1987). Thus, intensive agricultural systems like those in North America may be accelerating the evolution and speciation of *P. tritici-repentis*, along with that of other pathogens (Hansen 1987).

7.2 Occurrence of *ToxB* vs. *ToxA*

The fact that the *ToxA* gene is present only in tox^+ isolates led researchers to conclude that race differentiation in the fungus is likely due to the acquisition of toxin production by horizontal transfer (Ciufffeti et al. 1997). However, unlike what was found with *ToxA* (Ballance et al. 1996; Ciuffetti et al. 1997), the *ToxB* gene is present in many races, even some that were not known to produce the toxin (Fig. 4.2). Nevertheless, there appears to be a continuum of Ptr ToxB production and/or activity among isolates of *P. tritici-repentis*. The high toxin producers, for example race 6 isolates, are at one end of this continuum, followed by isolates such as 92-171R5, which possess only very low

levels of toxin activity (Fig. 3.1 and Fig. 3.2). Further down are isolates D308 and 90-2; both have the toxin gene, but they apparently possess fewer copies of it than isolates Alg 3-24, Alg-H2 and even 92-171R5 (Fig. 4.2). They express the gene almost at trace levels and possess no toxin activity. In the case of isolate 90-2, even the deduced amino acid sequence of the protein is no longer identical to that of the other isolates (Fig. 4.7). Finally, we have the isolates from races 1 and 2, in which the *ToxB* gene is completely absent. Thus, the situation with respect to Ptr ToxB appears more complex than is the case with Ptr ToxA. When it comes to Ptr ToxB, the mere fact that an isolate is tox$^-$ does not mean that the gene coding for the toxin is absent, or even transcriptionally silent.

7.3 Future Studies in Tan Spot

The tan spot system presents us with the opportunity to further our understanding of the evolution of pathogenicity and host-pathogen specificity, not only in the wheat/*P. tritici-repentis* interaction, but also in general. To understand what makes the toxins toxic, we must further characterize these virulence factors. The elucidation of the tertiary structures of Ptr ToxA and Ptr ToxB could provide us with valuable information as to their modes of action. The identification of receptors or targets for the toxins in the host cells could also prove invaluable in this respect. Once the receptors are known, we may be able to understand the exact basis for insensitivity or resistance in wheat. For Ptr ToxB, it may be useful to obtain genomic clones of *ToxB* from those isolates which transcribe it at very low levels; the information gained could be important in understanding why expression is so low. A multitude of other studies are possible, from investigations into the physiology of symptom development to quantitative expression

experiments, and all have the potential to provide important insights into the wheat/*P. tritici-repentis* interaction.

We must not, however, simply focus on biochemical or molecular aspects of the system. Most of the major breakthroughs to date in these areas have been possible because of similarly important breakthroughs in our understanding of the pathology of tan spot. For instance, if it were not for the recognition that chlorosis and necrosis are independent symptoms (Lamari and Bernier 1989a), it is doubtful whether any toxins would have ever been purified. Therefore, we should continue to identify and characterize new isolates and races. This will allow for the recognition of new pathogenicity factors, which could then be the focus of further biochemical, molecular and genetic studies. This will not only ensure progress in our understanding of the tan spot system, but also in our understanding of host-pathogen interactions in general.

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