University of Alberta

Heterologous expression and activity of Ptr ToxB from virulent and avirulent isolates of *Pyrenophora tritici-repentis*

by

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in
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Abstract

The fungal pathogen *Pyrenophora tritici-repentis* causes tan spot, a major foliar disease of wheat. The fungus produces at least three host-specific toxins, including the chlorosis-inducing toxin Ptr ToxB, a small protein encoded by the *ToxB* gene. To compare the activity of different forms of Ptr ToxB, *ToxB* from a virulent race 5 isolate (Alg3-24) and a *ToxB*-homolog from an avirulent race 4 isolate (90-2) were heterologously expressed as glutathione-S-transferase (GST)- and hexahistidine (His)-tagged fusion proteins in *Escherichia coli*. While the over-expressed GST- and His-tagged toxin proteins from Alg3-24 induced strong chlorosis symptoms in a genotype-specific manner, the Ptr ToxB-fusions from isolate 90-2 induced only trace amounts of symptoms. Therefore, it appears that the differences in amino acid sequence observed in Ptr ToxB from the avirulent isolate are sufficient to eliminate most of its activity. Furthermore, in an effort to gain insights into the mechanism of Ptr ToxB action, leaves of a toxin-sensitive wheat genotype were infiltrated with His-tagged toxin or water, and changes in the proteome compared 24 h later by 2-D gel electrophoresis. A total of 10 proteins were found to be up-regulated in the Ptr ToxB-treated tissue and subjected to ESI-Q-TOF MS/MS. The identities of six of these proteins were determined by MS/MS, and included proteins involved in stress/defense, photosynthesis and metabolism. Levels of the antioxidant enzyme superoxide dismutase (SOD) increased six-fold, suggesting an increase in reactive oxygen species (ROS) after treatment with the toxin. This finding seems to support previous research that suggested that Ptr ToxB-induced chlorosis may result from the formation of ROS, as a consequence of a direct or indirect inhibition of photosynthesis.

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1. Literature Review

1.1. Tan spot of wheat

Tan spot of wheat, also known as yellow leaf spot, is caused by the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.). Tan spot is characterized by two distinct symptoms: tan necrosis and extensive chlorosis. On susceptible wheat lines and cultivars, the pathogen produces oval-shaped lesions that have a necrotic center surrounded by a chlorotic border or halo (Wiese, 1987). Tan spot has been reported in all major wheat growing areas of the world. It occurs throughout North America, South America, Australia, North Africa, Europe, the Middle East, Azerbaijan, Kyrghyzstan, Kazakhstan, Uzbekistan, Syria, Afghanistan, Pakistan, and other parts of Asia (Hosford, 1982; Wiese, 1987: Lamari *et al.*, 2005a). Yield losses are quite significant when conditions are favorable for disease development, and generally range from 3 to 50% (Hosford, 1982; Rees *et al.*, 1982). Although commonly reported on wheat since the 1930's and 40's, it was not until the 1970's that the disease became a serious problem (Hosford, 1982). *Pyrenophora tritici-repentis* is a stubble-borne pathogen and the incidence of tan spot has increased in recent decades due to the adoption by farmers of agronomic practices that retain crop stubble, including minimum- and zero-tillage (Sutton and Vyn, 1990; Rees and Platz, 1992; Bailey, 1996). According to Rees and Platz (1992), however, changes in cultivar genotypes also played an important role in the increased incidence of tan spot, because much higher susceptibility to the

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disease was reported in semi-dwarf wheat varieties released since the 1960's. Nevertheless, in Canada at least, most hard red spring wheat cultivars planted through the 20th century were found to be susceptible to the necrosis and chlorosis induced by *P. tritici-repentis* (Lamari *et al.*, 2005b), and changes in cropping practices were therefore likely the most significant factor in increased tan spot prevalence.

1.1.1. Disease cycle of tan spot

Pyrenophora tritici-repentis overwinters on infected crop residues (stubble or straw). In spring, ascospores are dispersed by wind from the mature dark-brown sexual fruiting bodies of the fungus, the pseudothecia, and are generally considered to be the primary source of inoculum at this stage (Rees and Platz, 1983; Weise, 1987). However, a study from the Great Plains of the U.S.A. indicated that conidia produced on wheat debris from the previous year could also serve as a primary source of inoculum in spring wheat (Krupinsky, 1992b). In addition, Schilder and Bergstrom (1995) reported that seed transmission of the fungus can play a role as a primary source of infection by *P. tritici-repentis*. In western Canada, ascospore dispersal may be delayed because of prolonged cold temperature conditions (Bailey *et al.*, 2003). After dispersal of the ascospores, the lower leaves of young seedlings are infected by the pathogen, which penetrates the epidermis directly and grows intercellularly through the mesophyll of the leaf (Dushnicky *et al.*, 1996). Soon, lesions develop on susceptible plants under favorable conditions, such as wet weather and warm temperatures. Lamari and Bernier (1994) reported that temperatures between 20 °C and 25 °C are most favorable for infection, while disease development is delayed at

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temperatures under 20 °C and is inhibited at temperatures greater than 28 °C (Bailey *et al.*, 2003). After the primary infection by ascospores, asexual spores (conidia) are soon discharged from mature lesions, dispersed by the wind, and infect other leaves (Krupinsky, 1992b). Although ascospores of *P. tritici-repentis* are dispersed only over short distances, conidia may travel relatively longer distances (Schilder and Bergstrom, 1992). According to Hosford *et al.* (1987), air-borne ascospores and conidia require 12 h to 48 h for infection under moist conditions, and conidia can germinate after 6 h of wetness. Good growing conditions for the host are also favorable for the pathogen. Therefore, conidia produced in old lesions under favorable conditions become air-borne as leaves become dry, and play a role as a secondary source of inoculum during the growing season. After the secondary infection, the cycle will be repeated several times in a single growing season and the host will become more severely diseased (Bailey *et al.*, 2003).

1.1.2. Disease management

A variety of management strategies are recommended for tan spot control. These include cultural methods, such as crop rotation and tillage of the soil. A rotation to non-host crops (canola, corn, flax, potato and soybean) for at least 1 or 2 years (Bailey *et al.*, 2003; De Wolf *et al.*, 1998) is recommended under most conditions, to allow sufficient decomposition of the inoculum. Burning crop residues may also be effective for reducing inoculum sources, but it is not generally recommended since it reduces organic content in the soil. Burying crop residues before seeding (e.g. by working the soil) is effective in reducing the amount of air-borne inoculum (Bailey *et*

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al., 2003), and it is one of the main ways in which farmers prevented the build-up of inoculum in previous years. Nevertheless, this may not always be an effective strategy since spores can be transferred from other sources of inoculum by the wind. Unfortunately, all currently registered wheat cultivars grown in Canada are susceptible to tan spot (Bailey *et al.*, 2003), so use of genetically resistant wheat varieties is currently not a management option. Indeed, through most of the last century, large areas of Canada have been planted with genotypes susceptible to the necrosis and chlorosis induced by Ptr ToxA and Ptr ToxB (Lamari *et al.*, 2005b), which are important pathogenicity factors produced by *P. tritici-repentis* (Strelkov and Lamari, 2003).

Biological control may also have the potential to be a good management method, but it is hard to find effective biological antagonists of *P. tritici-repentis*. Pfender *et al.* (1993) reported that pseudothecial formation by the pathogen was suppressed by an antagonistic fungus, *Limonomyces roseipellis*, after it was sprayed on wheat crop residues in the field. *Limonomyces roseipellis* appears to be a mycoparasite that attacks the mycelium of *P. tritici-repentis*, restraining its reproduction system (Pfender *et al.*, 1993). However, the exact mode of action is poorly understood. Other fungi, such as *Myrothecium roridum*, *Stachybotrys* sp., and *Laetisaria arvalis*, also inhibit pseudothecial development by *P. tritici-repentis* (Pfender *et al.*, 1991). Nevertheless, successful biological control requires a combination of optimal timing of application of the control agent, complete coverage of the crop residue by the antagonist, adequate retention of the antagonist inoculum, and a choice of adequate

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formulation and application methods (Pfender *et al.*, 1993). This requirement makes use of effective biological control more challenging than other control methods, and may explain why this strategy has not been widely implemented in the field. Indeed, before biological control can be successful, it is necessary to understand the ecology of the residue environment and the mechanism of pathogen-antagonist interactions (Bockus, 1998).

Application of foliar fungicides, including protectant and systemic fungicides (Hamlen *et al.*, 1997), can provide successful control (Bailey *et al.*, 2003; Bockus, 1998). In Canada, foliar fungicides such as Headline EC (pyraclostrobin), Manzate DF (mancozeb), Dithane DG Rainshield NT (mancozeb), Penncozeb 75 DF (mancozeb), Bravo 500 (chlorothalonil), Stratego 250EC (propiconazole), Bumper 418EC (propiconazole), and Tilt 250E (propiconazole) are currently used (Celetti and Fernandez, 1998; Guide to Crop Protection 2006). However, it is important to consider the number and timing of applications, the fungicide formulation, the application method, and the cost of fungicide treatments. Finally, seed treatments and the use of healthy seeds are necessary components of an integrated management strategy. It is highly recommended not to propagate infected seeds (Celetti and Fernandez, 1998).

1.2. *Pyrenophora tritici-repentis*

Pyrenophora tritici-repentis was first isolated from *Agropyron repens* L. Beauv. (quack grass) and reported by Diedicke in 1902 (De Wolf *et al.*, 1998). The sexual stage (teleomorph) of the fungus was initially named *Pleospora tritici-repentis*

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Died. (Diedicke, 1903), but was renamed many times, and has been variously referred to as *Pleospora trichostoma* f. sp. *tritici-repentis* (Died.) Noack (1905), *Pyrenophora tritici-repentis* (Died.) Drechs. (1923), and *Pyrenophora tritici-vulgaris* Dickson (1956) (Hosford, 1982; De Wolf *et al.*, 1998). The asexual stage (anamorph) has been classified as *Helminthosporium graminearum* f. sp. *tritici-repentis* (Rab. Ex Schecht) Died. (1902), *H. tritici-repentis* (Died.) Died. (1903), *H. tritici- vulgaris* Nisikado (1928), *Drechslera tritici-vulgaris* (Nisikado) Ito. (1930), and *D. tritici-repentis* (Died.) Shoem. (1959) (Hosford, 1982; De Wolf *et al.*, 1998). In 1959, Shoemaker separated the genus *Helminthosporium* into three genera (*Drechslera*, *Bipolaris* and *Helminthosporium*) based on differences in conidial germination and included the tan spot fungus under *Drechslera* (De Wolf *et al.*, 1998). Shoemaker (1962) also concluded that the appropriate classification of the teleopmorph was in the genus *Pyrenophora*, after he identified ascospores and conidiophores with single conidia. Presently, the names *Pyrenophora tritici-repentis* (teleomorph) and *Drechslera tritici-repentis* (anamorph) are widely accepted (De Wolf *et al.*, 1998).

Pyrenophora tritici-repentis produces spherical black sexual fruiting bodies, pseudothecia, on over-wintered straw that contain numerous bitunicate, cylindric asci (Shoemaker, 1962). Ascospores are yellowish brown, biseriate (in two rows) in the centre and septate. The fungus also produces, at the asexual stage, light greenish to light brown conidia (usually 55 to 85 μm long) with cylindrical ends, narrowed at the apex and widest at the base, divided into five to seven cells by closed septa (Shoemaker, 1962). Conidiophores are formed in groups of about five, are light to

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medium brown, and have a slightly bent stalk (Shoemaker, 1962). *Pyrenophora tritici-repentis* has the widest host range of all species found in the genus *Pyrenophora* (Shoemaker, 1962). The pathogen can attack durum wheat (*Triticum turgidum* L. var. *durum*), common wheat (*Triticum aestivum* L.) and many other grass species including wheatgrass (*Agropyron* species and *Elymus* species), smooth bromegrass (*Bromus inermis* Leyss.), cereal rye (*Secale cereale*), wild rye (*Leymus* species), wild barley (*Critesion jubatum* (L.) Nevski), and wild oat (*Danthonia intermedia* Vasey) (Hosford, 1971; Krupinsky, 1992a; Ali and Francl, 2003). Indeed, Strelkov and Lamari (2003) hypothesized that *P. tritici-repentis* may have evolved on grasses before moving to its modern wheat host.

1.2.1. Pathotypes and races of *Pyrenophora tritici-repentis*

There have been many quantitative and qualitative attempts to describe and evaluate variation in isolates of *P. tritici-repentis*. Parameters including lesion size (Misra and Singh, 1972), percent leaf necrosis (Schilder and Bergstrom, 1990), and percent infection and lesion number (Luz and Hosford, 1980) were all proposed to discern variation in isolates of the pathogen. However, most of these quantitative systems met with limited success. The most widely used system for evaluating virulence in *P. tritici-repentis* was developed by Lamari and Bernier (1989a), and is based on a qualitative description of lesion type. Lamari and Bernier (1989a) observed that isolates of the fungus differed in their ability to produce necrosis or chlorosis symptoms on different hexaploid wheat genotypes. Therefore, they proposed that isolates of the fungus be grouped into four pathotypes according to their ability to

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cause these symptoms (Lamari and Bernier, 1989b): pathotype 1 isolates induce both necrosis and chlorosis (nec+chl+) on susceptible wheat genotypes, pathotype 2 isolates induce necrosis but not chlorosis (nec+chl–), pathotype 3 isolates induce chlorosis but not necrosis (nec–chl+), and pathotype 4 isolates induce neither symptom and are considered avirulent (nec–chl–) (Lamari and Bernier, 1989b).

The pathotype classification system was useful initially, but it had a major limitation - the fact that a maximum of only 4 pathotypes could be defined based on the presence or the absence of two symptoms. Furthermore, this system could not be used to differentiate isolates that produced the same symptom, but on different host genotypes. This limitation was highlighted with the discovery of pathotype 3 isolates from North Africa that induced chlorosis, but did so on different wheat lines/cultivars than pathotype 3 isolates from North America (Lamari *et al.*, 1995). Therefore, Lamari *et al.* (1995) proposed a race designation scheme, which could be used to classify an unlimited number of races based on the reaction of a specific set of wheat differential lines/cultivars. Using this classification scheme, at least eight races of *P. tritici-repentis* have been identified to date, according to their virulence on three effective wheat differentials, consisting of the cultivar Glenlea and lines 6B365 and 6B662 (Lamari *et al.*, 1995; Strelkov *et al.*, 2002; Lamari *et al.*, 2003). Race 1 isolates are able to attack 'Glenlea' and line 6B365, but not line 6B662. Race 2 isolates attack 'Glenlea' but are avirulent on 6B365 and 6B662. Race 3 isolates can attack 6B365, but are avirulent on 'Glenlea' and 6B662. Race 4 isolates cannot attack any of these differentials, and are considered avirulent. Race 5 isolates attack 6B662, but not 6B365

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or 'Glenlea' (Lamari *et al.*, 1995). In 2002, Strelkov *et al.* (2002) reported a new race, race 6, from North Africa that has a virulence pattern combining those of races 3 and 5, meaning that it can attack both 6B365 and 6B662. More recently, two new races were also identified by Lamari *et al.* (2003) from collections of the pathogen made in the Fertile Crescent and the Caucasus region of central Asia, the centers of diversity of wheat. Race 7 isolates show a virulence pattern that combines those of races 2 and 5. Thus, they can attack 'Glenlea' and line 6B662, but not line 6B365. Race 8 isolates combine the virulence patterns of races 2, 3, and 5, and can attack all three wheat genotypes (Lamari *et al.*, 2003). Therefore, according to the race classification system, races 1, 7, and 8 isolates are capable of producing both necrosis and chlorosis, race 2 isolates can only produce the necrosis symptom, races 3, 5, and 6 isolates cause chlorosis, but on different cultivars, and race 4 isolates are avirulent, producing neither symptom (Lamari *et al.*, 1995; Strelkov *et al.*, 2002; Lamari *et al.*, 2003). The susceptibility of the differential wheat genotypes to the different races of *P. tritici-repentis* is summarized in Table 1 (Strelkov and Lamari, 2003).

1.3. Host-specific toxins of *Pyrenophora tritici-repentis*

Phytotoxins are produced by many plant pathogenic fungi and bacteria and are compounds that can directly injure plant cells and influence disease or symptom development. Phytotoxins are classified as host-selective (syn. host-specific) or non host-selective (Pringle and Scheffer, 1964). Host-specific or host-selective toxins (HSTs) have high toxic activity toward only the hosts of the pathogen, whereas they

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Table 1. Host-specific toxins produced by the races of *Pyrenophora tritici-repentis* and the differential wheat genotypes that they affect.[1]

Cultivar or line	Race							
	4	2	1	8	7	5	6	3
'Glenlea'		Ptr ToxA						
6B662				Ptr ToxB				
6B365			Ptr ToxC				Ptr ToxC	
Number of toxins produced	0	1	2	3	2	1	2	1

[1] Adapted from Strelkov and Lamari (2003)

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show little or no toxic activity to non-host plants. Usually, HSTs are pathogenicity factors, in that disease occurs only in the presence of the toxins (Yoder, 1980). To date, HSTs from about 20 pathogens have been documented, most of which are low molecular weight secondary metabolites with diverse structures (Yoder, 1980; Walton, 1996). *Pyrenophora tritici-repentis* is known to produce at least three HSTs; two of these are unique among phytotoxins, because they are proteins encoded by single nuclear genes (Ballance *et al.*, 1989; Strelkov *et al.*, 1999). The nature of the Ptr toxins is summarized below.

1.3.1. Ptr ToxA

The existence of a host-specific toxic compound(s) produced *P. tritici-repentis* was first suggested by Tomas and Bockus (1987). They found that concentrated cell-free culture filtrates of certain virulent isolates of *P. tritici-repentis* produced necrosis symptoms similar to those caused by the fungus when infiltrated into susceptible cultivars. Therefore, Tomas and Bockus (1987) suggested the presence of a necrosis-inducing factor or toxin in these culture filtrates, and noted that they could be used in place of the fungus for identifying susceptible wheat lines and screening for tan spot resistance. Lamari and Bernier (1989c) also provided evidence for the production of a HST by *P. tritici-repentis*, which they characterized as a heat-labile molecule with a mass greater than 8,000 Da, and designated as Ptr necrosis toxin. This toxin was purified by the same research group and confirmed to be a heat-labile protein 13,900 Da in mass (Ballance *et al.*, 1989). Tomas *et al.* (1990) also purified the necrosis inducing toxin, and described it as a heat-stable protein with a mass of 14,700 Da,

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which they designated as Ptr toxin. In addition, Tuori *et al.* (1995) described a necrosis-inducing, heat-stable protein toxin with a 13,200 Da mass that they termed ToxA. Zhang *et al.* (1997) also purified a necrosis-inducing toxin of similar mass, and found it to have an isoelectric point near pH 10.

The host-sensitivity of these necrosis-inducing factors, the general properties of the molecules themselves (including the amino acid composition), and their generally similar size all strongly suggested that they were in fact the same toxin (Ciuffetti *et al.*, 1998). Discrepancies reported in the mass and heat stability likely resulted from differences in the experimental methodologies used to purify and/or characterize the toxin (Ciuffetti and Tuori, 1999). In an effort to avoid future confusion, tan spot workers from Canada and the U.S.A. agreed on a standardized nomenclature for the toxins of *P. tritici-repentis*, at the Third International Tan Spot Workshop, held in Winnipeg, Manitoba in 1997. Under this system, the necrosis-inducing toxin was renamed Ptr ToxA; as additional toxins are identified, they are assigned a different letter (e.g. Ptr ToxB, Ptr ToxC, etc.) in the order that they are reported (Ciuffetti *et al.*, 1998).

The gene, *ToxA*, responsible for Ptr ToxA production was cloned by two independent research groups, Ballance *et al.* (1996) and Ciuffetti *et al.* (1997). The open reading frame (ORF) of *ToxA* encodes a pre-pro-protein with a 19.7 kDa mass. The pre-pro-protein possesses a 22 amino acid signal peptide at the N-terminus, which is a common feature of secreted proteins. The precursor then undergoes a proteolytic cleavage by the fungus and is processed to yield the 13.2 kDa mature protein. The role

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of Ptr ToxA as a pathogenicity factor was confirmed by Ciuffetti *et al.* (1997), when they transformed an avirulent race 4 isolate of the pathogen with the *ToxA* gene, and showed that the transformed isolate was virulent on a Ptr ToxA-sensitive wheat cultivar. The interaction between Ptr ToxA and host plants has been studied by many researchers. Lamari and Bernier (1994) reported that cultivars sensitive to Ptr ToxA became insensitive to the toxin above 27 °C. They concluded that the failure of Ptr ToxA activity at higher temperatures likely resulted from conformational changes in the host target or receptor, which interferes with binding by the toxin (Lamari and Bernier, 1994). Kwon *et al.* (1996) also suggested that high temperatures (≥27 °C) may disrupt downstream signaling pathways involved in the toxin recognition mechanism. Moreover, Kwon *et al.* (1998) observed that Ptr ToxA-induced electrolyte leakage, an indication of toxin activity (Kwon *et al.* 1996), decreased after sensitive hosts were treated with sodium vanadate, an inhibitor of H^+-ATPase. Therefore, they concluded that the activity of Ptr ToxA requires an active host metabolism, including transcription, translation and active H^+-ATPase. More recently, Manning and Ciuffetti (2005) treated toxin-sensitive and insensitive wheat cultivars with Ptr ToxA, followed by protease K, an extracellular protease, and found that the toxin was protected in the leaves of sensitive, but not insensitive, cultivars They suggested that Ptr ToxA was protected from the protease either by association with a receptor or by internalization into host cells (Manning and Ciuffetti, 2005). The latter appears to be the case, since immunolocalization studies using green fluorescent protein (GFP)-tagged Ptr ToxA showed that the toxin is internalized into sensitive wheat mesophyll cells, without a

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disruption in the integrity of the plasma membrane (Manning and Ciuffetti, 2005). Once internalized, it appears that GFP-tagged Ptr ToxA localizes to cytoplasmic compartments and chloroplasts. Manning and Ciuffetti (2005) also demonstrated that the activity of Ptr ToxA is light dependent, but the exact mechanism involved in toxin action remains unknown (Manning and Ciuffetti, 2005). However, intracellular expression of the toxin in cells of sensitive and insensitive cultivars resulted in death of both types of cells, suggesting that the internal site of Ptr ToxA action is present in both genotypes (Manning and Ciuffetti, 2005).

1.3.2. Ptr ToxB

The second host-selective toxin (HST) to be identified from *P. tritici-repentis*, capable of inducing chlorosis in sensitive wheat lines and cultivars, was first identified from culture filtrates of a race 5 isolate of the fungus, and was originally designated Ptr chlorosis toxin (Orolaza *et al.*, 1995). It appeared to be hydrophilic protein with a mass of at least 3.5 kDa (Orolaza *et al.*, 1995). As per the nomenclature system adopted after the Third International Tan Spot Workshop, Ptr chlorosis toxin was later renamed Ptr ToxB (Ciuffetti *et al.*, 1998). Ptr ToxB was purified to homogeneity by Strelkov *et al.* (1999) and found to be a heat stable protein toxin, 6.61kDa in size, and active at concentrations as low as 14 nM. Ptr ToxB also appears to be a pathogenicity factor, *sensu* Yoder (1980). Information from a 30 residue partial N-terminus amino acid sequence (Strelkov *et al.*, 1999) enabled the gene encoding Ptr ToxB, termed *ToxB*, to be cloned by independent research groups (Fig. 1) (Martinez *et al.*, 2001; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006).

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(A)

```
Alg3-24 CTACAACTTTAAGAAAATGGCGCCTATATTCAAGACTACCAT 43
90-2    ----------------------------------------CCAT  4

Alg3-24 GCTACTTGCTGTGGCTATCCTCCCTGCTGCCCTTGTTTCGGCC 86
90-2    GCTACTTGCTGTGGCTATCCTTCCTGCTGCCCTTGTTTCGGCC 47

Alg3-24 AACTGCGTCGCCAATATCTTAAACATCAACGAAGCGGTT 125
90-2    AACTGTACCGCCAATATCTTGAACATCAACGAAGTGGTC 86

Alg3-24 ATTGCGACTGGCTGTGTCCCAGCTGGAGGGGAGCTTCGC 164
90-2    ATTGCGACTGGCTGTGTCCCAGCTGGAGGGAATCTTATC 125

Alg3-24 ATATTC GTGGGTAGT AGCCATAGCTATTTGATTAAGGCT 203
90-2    ATAAGGGTGGGCAGTGACCACAGCTATTTAATTAGGGCT 164

Alg3-24 ACAAGCTCCTGTGGTCTCAGCCTTA --- CTAATCAAGTA 239
90-2    ACAGT CTCCTGTGGTCTCAGCCTTAACCCCAGTCAATCA 203

Alg3-24 TTTATAAATGGCGAGAGTGTGCAAAGTGGAGGACGTTGTTAG 281
90-2    TTCATAAACGGCGAGAGTTTGGCAAGTGGAGGACGTTGTTAG 245
```

(B)

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Alg3-24 MAPIFKTTMLLAVAILPAALVSANCVANILNINEAVIATGCVPAG 45
90-2    -------- MLLAVAILPAALVSANCTANILNINEVVIATGCVPAG 45

Alg3-24 GELRIFVGSSHSYLIKATSSCGLSLTN-QVFINGESVQSGGRC 87
90-2    GNLIIRVGSDHSYLIRATVSCGLSLNPSQSFINGESLASGGRC 80
```

Figure 1. Nucleotide (A) and amino acid (B) sequence comparisons of *ToxB* and Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*. Sequences are identical except where indicated in the bold font. The signal peptide (B) or region of the ORF coding for the signal peptide (A) are underlined. The sequence data are available in GenBank for Alg3-24 (accession number AF483831) and 90-2 (accession number AF483832).

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In wild-type race 5 isolates, such as DW7 (North Dakota) and Alg3-24 (eastern Algeria), the *ToxB* gene contains a 261-bp ORF that encodes an 87 amino acid residue protein, which includes a 23 residue signal peptide. After cleavage of the signal peptide by the fungus, Ptr ToxB becomes a 64 residue long mature protein (Martinez *et al.*, 2001; Strelkov and Lamari, 2003). Unlike *ToxA*, which occurs as a single copy, *ToxB* is a multiple copy gene (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Furthermore, while *ToxA* is found only in isolates of the fungus possessing Ptr ToxA activity, homologs of the *ToxB* gene have been identified in isolates lacking Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006). For instance, the form of *ToxB* found in an avirulent race 4 isolate (90-2) of *P. tritici-repentis* is only 86% homologous to 'wild-type' *ToxB* from a highly virulent race 5 isolate (Alg3-24) over the ORF (Fig. 1) (Strelkov and Lamari, 2003). Recently, Strelkov *et al.* (2006) analyzed *ToxB* expression in Ptr ToxB producing and non-producing isolates of the fungus. Using a standard reverse-transcriptase (RT)-PCR protocol, they found that *ToxB* is expressed in mycelia and conidia of a wild-type race 5 isolate (Alg3-24), but only in conidia of Ptr ToxB non-producing isolates from races 3 (D308) and 4 (90-2), indicating differences in transcription of the gene (Strelkov *et al.*, 2006). Quantitative comparisons of expression levels and of in planta expression profiles are currently under way in our lab (S. Amaike and S.E. Strelkov, unpublished data). In addition, it appears that the *ToxB* gene is highly conserved among a geographically diverse set of isolates (Martinez *et al.*, 2004; Strelkov and Lamari, 2003: Strelkov *et al.*, 2006).

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To date, there has only been one study that examined the mode of action of Ptr ToxB. Strelkov *et al.* (1998) suggested that Ptr ToxB-induced chlorosis results from chlorophyll degradation, rather than an inhibition of chlorophyll synthesis, since they found that toxin-treatment did not interfere with chlorophyll accumulation in etiolated seedlings. Furthermore, they suggested that photochemical bleaching of the chlorophyll is likely involved in symptom development, as chlorosis is strictly light dependent. In addition, Strelkov *et al.* (1998) provided evidence that reactive oxygen species (ROS) are involved in this photochemical bleaching, since no chlorosis developed in toxin-treated tissues after incubation with certain ROS scavengers. Moreover, high performance liquid chromatography chlorophyll degradation profiles were consistent with photo-oxidation of the pigment (Strelkov *et al.*, 1998). Since carotenoids are important in dissipating the excess energy of chlorophyll molecules and in the detoxification of ROS, Strelkov *et al.* (1998) also examined the effect of Ptr ToxB on the carotenoid concentration of treated leaves. They found that the decline in carotenoid levels in sensitive tissue was concurrent with and of a smaller magnitude than the decline in chlorophyll, indicating that chlorosis does not develop as a result of a carotenoid deficiency. Strelkov *et al.* (1998) concluded that Ptr ToxB-induced chlorosis likely results from a direct or indirect inhibition of photosynthesis, resulting in the photo-oxidation of chlorophyll, as illuminated thylakoid membranes become incapable of dissipating excitation energy.

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1.3.3. Ptr ToxC

The third HST of *P. tritici-repentis*, designated Ptr ToxC, has been inferred from numerous studies (Lamari and Bernier, 1991; Gamba and Lamari, 1998; Gamba *et al.*, 1998), but has not been fully characterized. Like Ptr ToxB, Ptr ToxC induces chlorosis, but on different wheat genotypes. Effertz *et al.* (2002) partially purified Ptr ToxC from culture filtrates of a race 1 isolate of *P. tritici-repentis* and reported that it appears to be a polar, nonionic, low molecular weight compound. Hence, it is quite distinct from Ptr ToxA and Ptr ToxB, which are proteinaceous in nature (Effertz *et al.*, 2002). There are no published reports on the mode of action of this toxin, and the manner in which it induces chlorosis remains completely unknown. The production of host-specific toxins by the races of *P. tritici-repentis* is summarized in Table 1. In addition to Ptr ToxA, Ptr ToxB and Ptr ToxC, there are preliminary reports of the production of other HSTs by *P. tritici-repentis* (Manning *et al.*, 2002; Meinhardt *et al.*, 2003). However, these await further characterization.

1.4. Phytotoxins of other microorganisms

As noted above, many phytotoxins have also been identified from other plant pathogens. Given the importance of toxins in the development of tan spot of wheat, a few of the most well characterized toxins from other pathosystems are described below for the purposes of comparison. As with the Ptr toxins, most of these other toxins also cause the development of necrosis or chlorosis on sensitive host tissue.

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1.4.1. *Cochliobolus* HSTs

1.4.1.1. Victorin

Cochliobolus species belong to the Phylum Ascomycota and are common pathogens of monocots, a group which includes important crop species such as rice, maize and oat (Agrios, 1997). Victoria blight of oats is caused by the fungus *Cochliobolus* (*Helminthosporium*) *victoriae* (Meehan and Murphy, 1946), which produces the HST victorin, or HV toxin. Victorin is a chlorinated, cyclized-pentapetide, which generally induces changes in cell wall structure, loss of electrolytes from host cells, increased respiration, and decreased growth and protein synthesis (Agrios, 1997). Navarre and Wolpert (1995) reported that victorin binds to the glycine decarboxylase complex (GDC) of the mitochondria and inhibits GDC function. The GDC catalyzes the conversion of two glycine molecules into serine during the course of photorespiration. A recent study showed that victorin contributes to mitochondrial dysfunction (Curtis and Wolpert, 2002), which induces apoptosis of the cell.

1.4.1.2. T-toxin

T-toxin is a HST produced by *Cochliobolus heterostrophus* (*Helminthosporium maydis*; syn. *Bipolaris maydis*) race T isolates. *Cochliobolus heterostrophus* race T causes Southern corn leaf blight, a disease of maize carrying Texas cytoplasm for male sterility (Tcms). T-toxin is selectively toxic to Tcms maize and active at approximately 10 nM against maize with Tcms, while it is requires a concentration of at least 10 μM for activity against maize with normal cytoplasm. T-toxin is a mixture of long, linear polyketols (C35 to C41) (Yang *et al.*, 1996). This toxin specifically attacks the

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mitochondria of susceptible cells, which have a 13 kDa specific receptor protein molecule (URF13) localized to the inner mitochondrial membrane (Wise *et al.*, 1987). URF13 is a polypeptide tetramer located in the mitochondrial membrane and has been implicated in causing male sterility. When T-toxin directly binds to URF13, it undergoes a conformational change that leads to the formation of a pore in the mitochondrial membrane by the toxin/URF13 complex (Levings *et al.*, 1995). Pore formation causes loss of mitochondrial integrity and leads to leakage of cofactors, substrates, and protons, which eventually induces cell death.

1.4.1.3. HC-toxin

The filamentous fungus *Cochliobolus carbonum* (*Helminthosporium carbonum*; syn. *Bipolaris zeicola*) causes Northern leaf spot and ear rot of maize, and produces the host selective HC-toxin. HC-toxin belongs to a family of cyclic tetrapeptides produced by a 570-kDa non-ribosomal peptide synthetase called HC-toxin synthetase (HTS) (Scott-Craig *et al.*, 1992). HC-toxin inhibits histone deacetylase (HD) in susceptible maize (Brosch *et al.*, 1995). Treatment of susceptible maize with HC-toxin resulted in histone hyperacetylation (Ransom and Walton, 1997). However, Brosch *et al.* (1995) reported that interference with histone acetylation is not lethal to cells, but rather interferes with embryo development and normal gene regulation. Therefore, one possible explanation of the action of HC-toxin is that it may permit infection by interfering with the induction of maize defense genes (Brosch *et al.*, 1995). From this perspective, Walton and Panaccione (1993) noted that HC-toxin might be better defined as a suppressor rather than as a toxin.

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1.4.2. *Alternaria* HSTs

Fungi belonging to the genus *Alternaria* produce many secondary metabolites that function as HSTs. For example, *A. alternata* f.sp. *fragariae* infects strawberry and produces AF-toxin. *Alternaria alternata* f.sp. *citri* infects tangerine and produces ACT-toxin, which acts at the plasma membrane of host cells (Otani *et al.*, 1995). AM-toxin is produced by *A. alternata* f.sp. *mali*, the cause of Alternaria leaf blotch (necrosis) in susceptible apple genotypes. AM-toxin is a four-member cyclic depsipeptide (Okuno *et al.*, 1974) that acts on both the plasma membrane and chloroplasts. Additional toxins produced by members of the genus *Alternaria* are discussed below.

1.4.2.1. AAL-toxins

AAL-toxins are HSTs produced by *Alternaria alternata* f. sp. *lycopersici* (Gilchrist and Grogan, 1976), which causes Alternaria stem canker of tomato (Grogan *et al.*, 1975). AAL-toxins have 5 different forms (AAL-toxin TA, TB, TC, TD, and TE; each with two isomers). The AAL-toxins are related to a larger group of compounds that includes sphingosine and sphinganine, and are referred to as sphinganine analog mycotoxins (SAMs). AAL-toxins induce an apoptotic-like response in toxin-sensitive tomato (Wang *et al.*, 1996). This toxin-induced apoptosis may involve the inhibition of ceramide synthase, an enzyme in the sphingolipid synthesis pathway (Wang *et al.*, 1991), resulting in accumulation of free sphingoid bases such as phytosphingosine (sphingosine) and sphinganine. Sphingolipids are major structural components in plasma membranes. Therefore, lesions may result from

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disruption of sphingolipid metabolism via inhibition of ceramide synthase (Abbas *et al.*, 1994).

1.4.3. Fungal non host-selective phytotoxins

Unlike HSTs, which are crucial for disease development and are considered pathogenicity factors, non host-selective toxins tend to serve as virulence factors, in that they may increase symptom severity and aid in infection, but are not indispensable for disease development. Furthermore, unlike most HSTs, non host-selective toxins can induce symptoms on plant genotypes that do not serve as hosts for the producing pathogen. Some of the most well characterized non host-selective toxins are described below.

1.4.3.1. Fusicoccin

The fungus *Fusicoccum amygdali* produces the non host-selective toxin, fusicoccin. This pathogen generally causes wilting in peach and almond, but can also attack higher plants (Marré, 1979). Fusicoccin is a diterpenoid glycoside synthesized via the mevalonic acid pathway. The toxin targets plasma membrane-localized H^+-ATPase. The plasma membrane-localized receptors for fusicoccin are 14-3-3 proteins that are present in all eukaryotes and act as regulators in various signal transduction pathways (Korthout and De Boer, 1994). After the plant H^+-ATPase is activated by fusicoccin, increased uptake of Cl^-, K^+ (and other cations), and water induces the irreversible opening of the stomatal guard cells and increases transpiration, eventually causing wilting of affected leaves (Ballio, 1991).

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1.4.3.2. Tentoxin

Tentoxin is produced by the fungus *Alternaria alternata* (formerly *Alternaria tenuis*). The pathogen causes chlorosis in many species of seedling plants (Agrios, 1997). Tentoxin is a cyclic tetrapeptide (Agrios, 1997) that targets and inactivates the αβ subunit complex of the chloroplast coupling factor 1, which is involved in energy transfer into chloroplasts. The toxin also inhibits the light-dependent photophosphorylation of ADP to ATP, resulting in chlorosis by a disruption in chlorophyll biosynthesis (Knogge, 1996).

1.4.4. Non host-specific phytotoxins of *Pseudomonas syringae*

Pseudomonas syringae is a bacterium that causes a broad range of symptoms on a number of hosts, including blights, leaf spots and galls (Agrios, 1997). The phytotoxins produced by *P. syringae* generally induce necrosis (syringomycin and syringopeptin) or chlorosis (coronatine, phaseolotoxin, and tabtoxin) (Bender *et al.*, 1999). These toxins are also secondary metabolites and include monocyclic β-lactam (tabtoxin), sulfodiaminophosphinyl peptide (phaseolotoxin), lipodepsinonapeptide (syringomycin), lipodepsipeptide (syringopeptin), and polyketide (coronatine) structures (Mitchell, 1991).

1.4.4.1. Syringomycin

Syringomycin is produced by most strains of *P. syringae* pv. *syringae*, which has a wide range of host plants and causes citrus blast, pear blast, bean leaf spot, and lilac blight (Agrios, 1997). Syringomycin is a cyclic lipodepsinonapeptide phytotoxin,

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which is composed of a polar peptide head and a hydrophobic 3-hydroxy fatty acid tail (Segre *et al.*, 1989). Syringomycin causes necrosis in plant tissues. Backman and DeVay (1971) reported that the plasma membrane of host cells is the primary target of syringomycin action. The lipopeptide structure of syringomycin promotes its insertion into the lipid bilayers of membranes to form pores (Hutchison *et al.*, 1995). Sphingolipids, major lipid components of eukaryotic plasma membranes, are associated with sensitivity to syringomycin (Grilley *et al.*, 1998). Pore formation by the toxin causes an increase in transmembrane fluxes of K^+, H^+ and Ca^{2+} that are deadly to cells (Mott and Takemoto, 1989).

1.4.4.2. Syringopeptin

Syringopeptin is also produced by *P. syringae* pv. *syringae* and belongs to the lipodepsipeptide family of phytotoxins (Ballio *et al.*, 1991). Like syringomycin, syringopeptin also forms pores in plant plasma membranes, causing electrolyte leakage in affected cells (Iacobellis *et al.*, 1992). By forming pores in the plasma membrane, it promotes trans-membrane ion fluxes and induces necrosis, which results in cell death. Therefore, syringomycin and syringopeptin are both pore-forming phytotoxins that cause necrosis in plants by a similar mechanism (Hutchison and Gross, 1997).

1.4.4.3. Coronatine

Coronatine (COR) is a non host-specific phytotoxin produced by several different pathovars of *P. syringae*. COR is composed of two distinct structural components: the polyketide coronafacic acid (CFA) and coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Ichihara *et al.*, 1977; Mitchell,

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1985; Parry *et al.*, 1994). Both CFA and CMA must be joined together by an amino bond to form the active toxin. Coronatine induces diffuse chlorosis in a wide variety of plant species (Gnanamaickam *et al.*, 1982). The structure of COR is similar to methyl jasmonate (MeJA), a compound produced in many plants in response to stress that acts as a growth regulator (Wasternack and Parthier, 1997). Both COR and MeJA induce the production of proteinase inhibitors; however, only COR causes thickening of cell walls, changes in chloroplast structure (Palmer and Bender, 1995), and chlorosis resulting from chlorophyll degradation (Kenyon and Turner, 1990).

1.4.4.4. Tabtoxin

Tabtoxin is a chlorosis-inducing phytotoxin produced by *P. syringae* pv. *tabaci*, which causes wildfire disease of tobacco (Agrios, 1997). Tabtoxin is a dipeptide toxin containing tabtoxinine-β-lactam (TβL), which is linked by a peptide bond to threonine (Mitchell, 1991). Tabtoxin itself is not a toxin, but the chlorosis-inducing activity occurs only after hydrolysis of the peptide bond by aminopeptidases of plant or bacterial origin, which releases TβL, thereby resulting in the active toxin (Bender *et al.*, 1999). TβL is toxic to cells because it irreversibly inhibits the enzyme glutamine synthetase, which synthesizes glutamine (Bender *et al.*, 1999). This results in decreased glutamine levels and increased ammonia levels. Ammonia is toxic to plants because it disrupts the thylakoid membranes of the chloroplast and uncouples photosynthesis and photorespiration (Turner and Debbage, 1982), thereby resulting in chlorosis.

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1.4.4.5. Phaseolotoxin

Phaseolotoxin is also a chlorosis-inducing phytotoxin, produced by *P. syringae* pv. *phaseolicola*, the cause of halo blight of legumes (Mitchell, 1976). Phaseolotoxin consists of a modified ornithine-alanine-homoarginine tripeptide carrying a phosphosulfinyl group (Mitchell, 1976). Phaseolotoxin entirely inhibits the activity of the enzyme ornithine carbamoyltransferase (OCTase), which is a critical enzyme in the urea cycle and converts ornithine and carbamoyl phosphate to citrulline, a precursor of arginine (Bender *et al.*, 1999). The inactivation of OCTase causes accumulation of ornithine and a deficiency in intracellular pools of arginine and citrulline. This results in a protein deficiency and reduced chlorophyll biosynthesis in affected tissues, leading to chlorosis (Bender *et al.*, 1999).

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1.5. Research objectives

Different forms of Ptr ToxB have been identified in virulent and avirulent isolates of *P. tritici-repentis*. The form of the toxin found in avirulent race 4 isolates of the fungus is only 86% homologous to 'wild-type' toxin from highly virulent race 5 isolates (Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Differences in the activity of these different forms of Ptr ToxB could help explain the decreased virulence observed in certain isolates. Thus, the primary objective of the present research was to optimize the heterologous expression of Ptr ToxB from races 4 and 5 in *Escherichia coli*, in order to compare the activities of the different forms of the toxin. We hypothesized that the activity of these two forms of the toxin would differ and be greatest in the wild-type. In addition, an optimized system for Ptr ToxB expression in *E. coli* will facilitate future research, by enabling rapid production and purification of the toxin, thereby eliminating the need to purify it from culture filtrates of the fungus. A secondary objective of the research was to conduct a preliminary characterization of the proteome-level changes induced by Ptr ToxB in toxin-sensitive tissue. We hypothesized that treatment with the toxin would induce changes in the proteome that would be detectable prior to chlorosis symptom development. Information obtained by comparing the proteomes of treated and untreated leaf tissue could provide important clues as to the mechanism of action of Ptr ToxB.

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2. Heterologous expression and activity of Ptr ToxB from virulent and avirulent isolates of *Pyrenophora tritici-repentis*

2.1. Introduction

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), is an economically important foliar disease of wheat. Tan spot occurs throughout the major wheat growing regions of the world and results in yield losses ranging from 3 to 50% (Hosford, 1982). Since *Pyrenophora tritici-repentis* is a stubble-borne pathogen, disease incidence has increased in recent decades as farmers have adopted soil conservation practices that retain crop residues (Bokus and Classsen, 1992; Bailey, 1996). Tan spot is characterized by the development of two distinct symptoms, tan necrosis and extensive chlorosis, which are differentially induced by host-specific toxins (HSTs) produced by the fungus. Currently, at least three HSTs are known. Ptr ToxA (formerly known as Ptr toxin, Ptr necrosis toxin and ToxA [Ciuffetti *et al.*, 1998]) induces necrosis on sensitive wheat lines/cultivars, and is a 13.2 kDa protein encoded by a single copy gene, *ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997; Ciuffetti *et al.*, 1998). The *ToxA* gene encodes a 19.7 kDa preproprotein that contains a 22 amino acid (aa)-residue signal peptide, which undergoes proteolytic cleavage to give a mature protein 118 aa in length (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Ptr ToxB (formerly Ptr chlorosis toxin [Ciuffetti *et al.*, 1998]) induces chlorosis on sensitive wheat lines/cultivars, and is also a small protein, 6.61 kDa in mass (Strelkov *et al.*, 1998).

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This toxin, which is encoded by the *ToxB* gene, possesses a 23 aa-residue signal peptide that is cleaved to give a mature protein 64 aa in length (Martinez *et al.*, 2001; Strelkov and Lamari, 2003). Unlike Ptr ToxA and Ptr ToxB, the third HST produced by *P. tritici-repentis*, termed Ptr ToxC, is not a protein, but instead appears to be a polar, non-ionic, low-molecular-mass molecule (Effertz *et al.*, 2002). Although it also induces chlorosis, it does so on different host genotypes than Ptr ToxB. In addition to these toxins, there are preliminary reports of at least two other HSTs produced by *P. tritici-repentis* (Manning *et al.*, 2002; Meinhardt *et al.*, 2003), but these await further characterization.

Whereas the *ToxA* gene is found as a single copy only in Ptr ToxA-producing isolates of *P. tritici-repentis* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997), the *ToxB* gene is present in multiple copies in Ptr ToxB-producing (Ptr ToxB+) isolates, which represent races 5, 6, 7 and 8 of the fungus (Martinez *et al.*, 2001; Strelkov, 2002; Lamari *et al.*, 2003; Strelkov *et al.*, 2003). However, in addition to its occurrence in Ptr ToxB+ isolates, single copies of a *ToxB* homolog have also been identified in races 3 and 4 isolates of *P. tritici-repentis*, which do not possess any detectable Ptr ToxB activity (Ptr ToxB-) (Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Analysis of the form of *ToxB* (termed *toxb* by Martinez *et al.*, 2004) found in avirulent race 4 isolates revealed 86% homology over the open reading frame (ORF) relative to 'wild-type' *ToxB* from race 5 isolates (Strelkov and Lamari, 2003; Martinez *et al.*, 2004). However, although this form of the toxin does not have any known biological function, the *ToxB* homolog is identical amongst race 4 isolates, even those

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with quite different geographic origins (Strelkov and Lamari, 2003; Martinez *et al.*, 2004). It also appears to be an active gene, since *ToxB* transcript was detected in conidia of race 4 isolate 90-2, albeit at a much lower level than in race 5 isolate Alg3-24 (Strelkov *et al.*, 2006). Therefore, it is not clear if the lack of Ptr ToxB activity in race 4 isolates results from differences in the gene sequence and/or level of expression relative to wild-type *ToxB*. In an effort to answer this question, and to further elucidate the role of Ptr ToxB and its related forms in symptom development and fungal virulence, we cloned and expressed the *ToxB* homologs from races 4 and 5 in an *Escherichia coli* expression system, and compared their chlorosis-inducing activity on toxin-sensitive and insensitive wheat genotypes.

2.2. Materials and Methods

2.2.1. Fungal isolates

Isolates 90-2 and Alg3-24 of *P. tritici-repentis*, representing races 4 and 5, respectively, of the pathogen (Lamari and Bernier, 1989; Lamari *et al.*, 1995), were selected for use in this experiment. Both isolates were initially grown in darkness on V8-potato dextrose agar (Lamari and Bernier, 1989) at 20 °C until the colony diameter reached 5 cm. To induce sporulation, mycelia were crushed using a sterile test tube bottom and incubated under light at room temperature (RT) for 18 h, and then in darkness at 15 °C for 24 h (Lamari and Bernier, 1989). Conidia were harvested in sterile distilled water and quantified using a hemocytometer. A 2 mL sample of

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conidial suspension (approximately 2 × 10^4 conidia/mL) from each isolate was transferred to 200 mL of Fries' medium amended with 0.1% yeast extract (Dhingra and Sinclair, 1986). Liquid cultures were incubated in darkness for 3 weeks without agitation, and mycelial mats were harvested by vacuum filtration through Whatman grade No.1 filter paper (Whatman International Ltd., Maidstone, UK). The mycelial mats were flash frozen in liquid N_2, lyophilized and stored at -80 °C for DNA extraction.

2.2.2. Plant material

The wheat cultivars Katepwa (sensitive to Ptr ToxB) and Glenlea (insensitive to Ptr ToxB) were grown in plastic pots (12.7 cm in diameter) filled with Metro Mix® 220 (Grace Horticultural products, Ajax, ON) at a density of 5 seedlings per pot. Plants were grown to the 3-4 leaf stage and maintained in a growth cabinet at 21 °C (day) and 18 °C (night) with a 16 h photoperiod at a light intensity of 180 μmol photons $m^{-2} s^{-1}$. Seedlings were watered and fertilized as required. Experiments were repeated three times, and treatments were replicated three times in each experiment.

2.2.3. DNA extraction

Genomic DNA was extracted from 40 mg samples of lyophilized mycelium using cetyltrimethylammonium bromide (CTAB), according to the method of Rogers and Bendich (1994). Extracted genomic DNA was digested with *Hin*dIII (Sigma-Aldrich, Oakville, ON) and further treated with RNase A (10 mM Tris-HCl, pH 7.5, 15 mM NaCl) (Sigma-Aldrich) at 37 °C for 2 h to remove RNA contamination.

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2.2.4. Protein estimation and electrophoresis

Protein concentration was determined according to the method of Bradford (1976) using a commercial kit (Bio-Rad, Mississauga, ON), with bovine serum albumin included as a standard. Polyacrylamide gel electrophoresis (PAGE) was carried out under denaturing conditions with sodium docecyl sulfate (SDS), in a Mini-PROTEAN® 3 cell apparatus (Bio-Rad), using the buffer system of Schagger and von Jagow (1987). Proteins were separated on a 10% separating gel and 5% stacking gel, of 0.75 mm thickness. After running, the gels were fixed and stained with 0.05% (w/v) Coomassie brilliant blue R-250 in methanol-acetic acid-water (5:1:4, v/v/v) for 30 min, and destained in methanol-acetic acid-water (0.5:0.7:8.8, v/v/v). The expected sizes of the fusion proteins were calculated using the Compute pI/Mw software program (Bjellqvist *et al.*, 1993).

2.2.5. Primers

The primers used to amplify the *ToxB* gene were designed based on sequence data available in GenBank for Alg3-24 (accession no. AF483831) and 90-2 (accession no. AF483832), using Primer3 software (Rozen and Skaletsky, 2000). Forward primer YMK1F (5'-AA AAA GCA GGC TTC GAA AAC CTG TAT TTT CAG GGC **GTG GCT ATC CTC CCT GCT G**-3') and reverse primer YMK1R (5'-A GAA AGC TGG GTC **GGA AAA CTG TGC TAT GCC AGA**-3') were used to amplify a 225 bp region of the *ToxB* ORF from the virulent isolate Alg3-24. Forward primer YMK2F (5'-AA AAA GCA GGC TTC GAA AAC CTG TAT TTT CAG GGC **CTC CCT GCT GCC CTT GTT**-3') and reverse primer YMK2R (5'-A GAA AGC TGG GTC

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AAT CTT GTA GCG CCT AAA CTC TGT T-3') were used to amplify a 219 bp region of the ORF from the *ToxB* homolog found in the avirulent isolate 90-2. Gene-specific primer sequences are noted in bold text, while TEV protease cleavage sites are underlined. To enable subsequent insertion of the amplified fragments between the *att*P1 and *att*P2 sites (Landy, 1989) of the pDONR^{TM} 221 vector (Invitrogen, Carlsbad, CA), a portion of the *att*B1 and *att*B2 adapter sequences was included at the 5'-ends of the forward and reverse primers, respectively. These served as templates for amplification by a second set of primers, consisting of forward primer *att*B1 (5'-G GGG ACA AGT TTG TAC AAA AAA GCA GGC T-3') and reverse primer *att*B2 (5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3'), as described below.

2.2.6. PCR Amplification

The *ToxB* ORF from each isolate was amplified according to the GATEWAY™ Cloning Techonology Adapter PCR Protocol (Invitrogen) to enable cloning of the PCR products into the pDONR^{TM} 221 vector (Invitrogen). In the first step of this two step protocol, PCR amplifications were conducted using the gene-specific primers YMK1F/YMK1R or YMK2F/YMK2R in a 50 µL reaction volume, consisting of 200 nM of each forward and reverse primer, 0.2 mM of each dNTP, 500 ng of DNA template, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, and 2U Platinum® *Taq* DNA polymerase. The amplification cycle consisted of an initial heat denaturation step at 95 °C for 2 min, followed by 10 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 68 °C for 30 sec. In the second step of the protocol, 5 µL from the first PCR reaction mixture were added to a 50 µL reaction volume containing

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800 nM of each primer (*att*B1 and *att*B2), 0.2 mM of each dNTP, 1× PCR buffer, 1.5 mM $MgCl_2$, and 2U Platinum® *Taq* DNA polymerase. Following an initial 1 minute denaturation step at 95 °C, 5 amplification cycles were performed of 94 °C for 15 sec, 45 °C for 30 sec, and 68 °C for 30 sec, followed by an additional 20 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 68 °C for 30 sec. All PCR amplifications were conducted using a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA).

Amplicons were resolved on ethidium bromide-stained 1% (w/v) agarose gels in 1× Tris-acetate-EDTA buffer (40 mM Tris; 0.1% [v/v] glacial acetic acid; 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). The size of the amplification products was confirmed using gel documentation software (Gel Doc 1000, Bio-Rad), and the corresponding bands excised from the gels under UV light. The products were extracted from the gel pieces using Ultrafree-DA Centrifugal Filter Devices (Millipore Canada Ltd., Mississauga, ON) according to the manufacturer's instructions, and further purified by extraction in a 1:1 (v/v) mix of phenol/isoamyl alcohol [24:1 (v/v)]:phenol, to which a $1/100^{th}$ volume of Quick-Precip™ Solution was added (Edge BioSystems, Gaithersburg, MD).

2.2.7. Transformation

Purified amplicons containing the *att*B1 and *att*B2 attachment sequences were inserted between the *att*P1 and *att*P2 recombination sites of the pDONR™ 221 vector and transformed into Library Efficiency® DH5α™ *E. coli* cells (Invitrogen), as per the manufacturer's instructions. Transformants were selected against 50 μg/mL

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kanamycin. Positive controls were transformed with the pEXP7-tet vector (Invitrogen) encoding the tetracycline resistance gene, while negative controls were subjected to the transformation protocol minus the plasmid. Entry vectors carrying the *ToxB* insert were purified from 3 mL of bacterial culture using a commercial kit (Qiagen, Mississauga, ON), and sequenced at the Molecular Biology Service Unit, University of Alberta, on a 3730 DNA Analyzer (Applied Biosystems). The pDEST™ 15 and pDEST™ 17 vectors (Invitrogen) were used for expression of glutathione-S-transferase (GST)- and hexahistidine (His)-fusion proteins, respectively. The *ToxB* constructs were inserted between the *att*R1 and *att*R2 recombination sites of the expression vectors via the site-specific recombination system of bacteriophage lambda (Landy 1989), according to the manufacturer's protocol, and transformed into BL21-AI™ *E. coli* cells (Invitrogen). Transformed cells were selected against 100 μg/mL ampicillin. Positive controls were transformed with the pENTR™-gus vector (Invitrogen) encoding the *Gus* gene, while no plasmid was included when transforming negative controls. As a final confirmation that the expression clones contained the correct inserts, colony PCR (Güssow and Clackson, 1989) was conducted using the *ToxB*-specific primers YMK1F/YMK1R or YMK2F/YMK2R, with an amplification cycle consisting of 94 °C for 5 min, 35 cycles of 94 °C for 15 sec, 55 °C for 30 sec, 68 °C for 30 sec, and a final extension step of 68 °C for 10 min.

2.2.8. Protein expression

For small scale expression of the Ptr ToxB constructs, transformed *E. coli* cells were initially cultured in 5 mL of Luria-Bertani (LB) medium (Sambrook *et al.*, 1989),

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containing 100 μg/mL ampicillin, at 37 °C with shaking (250 rpm) for 5 h. A 1 mL aliquot from each culture was used to inoculate 19 mL of fresh LB medium, containing 100 μg/mL ampicillin, and incubated as above until the cells reached the mid-log phase. Fusion protein expression was initiated by the addition of L-arabinose (Sigma-Aldrich) to a final concentration of 0.2% (w/v), after which the cultures were incubated for another 4 h or overnight (O/N). Five hundred μL aliquots from each culture were collected at various time points (0 h, 1 h, 2 h, 3 h, 4 h or O/N) to assess protein expression levels. Bacterial cells were harvested by centrifugation at 16,000 × g for 10 min at RT and the supernatants discarded. The cell pellets were re-suspended in a total volume of 150 μL lysis buffer (10 mM phosphate buffered saline [PBS], pH 7.4; 500 μg/mL lysozyme; 5 mM dithiothreitol [DTT]; 1 mM phenylmethylsulphonylfluoride [PMSF]; 5 μg/mL DNase I; 5 μg/mL RNase A; 1.5% sarkosyl, [w/v]; 1% [v/v] Triton® X-100) and incubated on ice for 30 min with occasional vortexing. The samples were centrifuged at 16,000 × g for 5 min at RT and the pellets and supernatants analyzed for fusion protein expression and solubility by SDS-PAGE. For large scale protein expression, a 1 mL volume of each initial *E. coli* culture was used to inoculate 250 mL of fresh LB medium containing 100 μg/mL ampicillin, with subsequent steps conducted as above, except that bacterial cells were harvested by centrifugation at 7000 × g for 10 min at 4 °C.

2.2.9. Isolation of proteins

The cell pellets from 160 mL samples of culture were re-suspended in 20 mL cell lysis buffer (10 mM PBS, pH 7.4, 500 μg/mL lysozyme, 1 mM PMSF, 5 mM DTT

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or 20 mM β-mercaptoethanol [βME], 10 μg/mL DNase I, 10 μg/mL RNase A and 1% [v/v] Triton X-100), incubated on ice for 30 min with occasional gentle shaking, and sonicated in a water bath for 2 min. The resulting homogenates were centrifuged at 27,000 × g for 15 min at 4 °C and the supernatants containing the soluble protein fraction removed. For purification of GST- or His-tagged Ptr ToxB, the insoluble protein fraction (pellet) was re-suspended in denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea and 20 mM βME, pH 8.0) and incubated at RT with stirring for 1 h. Insoluble materials were removed by centrifugation at 27,000 × g for 5 min at 20 °C, while proteins in the re-solubilized fraction were refolded in vitro as described below. His-tagged recombinant Ptr ToxB was batch purified by affinity chromatography prior to the refolding step. Briefly, 20 mL samples of the re-solubilized protein fraction were separated into 10 mL aliquots and incubated with shaking (200 r/min) at RT for 1 h in a 6 mL slurry (50%, w/v) of nickel-nitrilotriacetic acid (Ni-NTA) agarose resin in 30% (v/v) ethanol (Qiagen). The samples were then loaded onto Poly-Prep chromatography columns (Bio-Rad) and washed 3× with 5 mL wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea and 30 mM imidazole, pH 8.0) under gravity flow. The His-tagged Ptr ToxB was eluted from the resin in 10 mL of elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea and 300 mM imidazole, pH 8.0) under gravity flow and collected for refolding.

2.2.10. Refolding of fusion proteins

The re-solubilized GST-tagged and batch purified His-tagged Ptr ToxB proteins were refolded in vitro. Ten mL protein samples were placed in 1,000

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molecular mass cut-off dialysis tubing (Spectra/Pro®, Spectrum Laboratories Inc., Rancho Dominguez, CA) and dialyzed against 1 L of 6 M urea (Fisher Scientific Ltd., Ottawa, ON) for 6 h at 4 °C with gentle stirring. A 2 L volume of 25 mM Tris-HCl (pH 7.5) buffer was gradually added to the 6 M urea buffer, in 250 mL or 500 mL aliquots, over 48 h until the final volume reached 3 L. Protein samples were then subjected to a final dialysis step against 2 L of 12.5 mM Tris-HCl, 0.125 mM EDTA (pH 8.0) or 5 mM sodium acetate (pH 4.6) buffer for 12 h at 4 °C. Precipitates formed during dialysis were removed by centrifugation at 16,000 × g at 4 °C for 5 min and the supernatants transferred to new tubes. The dialyzed protein samples were concentrated 4× by freeze-drying and digested with rTEV protease enzyme (Invitrogen), as per the manufacturer's instructions, to remove the GST- or His-tags. All refolded fusion protein samples as well as the flow-through, washes, precipitates, and digested proteins were analyzed by SDS-PAGE for the presence of Ptr ToxB.

2.2.11. Bioassays

Bioassays were conducted by infiltrating the second and third leaves of wheat seedlings at the 3 to 4 leaf stage with 500 µL samples of GST-. or His-tagged recombinant Ptr ToxB, in 50 mM Tris-HCl, 0.5 mM EDTA (pH 8.0) or 20 mM sodium acetate (pH 4.6) buffer, using a Hagborg (1970) device. The activity of the various heterologously expressed proteins was tested at concentrations of 1 nM, 10 nM, 0.1 µM, 0.2 µM, 0.5 µM, 1 µM or 2 µM concentrations. Controls were infiltrated with buffer.

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2.2.12. Estimation of pigment concentrations

Leaf tissue segments (40 mg total weight), randomly chosen from each treatment, were cut, weighed and frozen in liquid nitrogen. They were ground in a mortar and pestle and extracted twice with 600 µL volumes of 80% acetone (v/v). The extracts were transferred to clean 1.5 mL microcentrifuge tubes and vortexed vigorously for several seconds. They were then incubated in darkness at RT for 20 to 25 min with occasional vortexing and centrifuged at 16,000 × g for 5 min. The supernatants were transferred into 15 mL Falcon tubes (Becton Dickinson Labware, Franklin Lakes, NJ), and the pellets (plant residues) remaining in the microcentrifuge tubes were extracted twice more with 1 mL volumes of 80% acetone (v/v). The supernatants were combined in the 15 mL Falcon tubes and the final volume adjusted to 4 mL with 80% acetone (v/v), corresponding to a final concentration of 1 mL acetone per 10 mg (fresh weight) of leaf tissue extracted (Witham *et al.*, 1971). Samples were measured spectrophotometrically at 470, 647 and 663 nm and chlorophyll and carotenoid concentrations calculated according to the equations of Lichtenthaler (1987).

2.2.13. Thermal stability

The heat stability of His-tagged Ptr ToxB from Alg3-24 and 90-2 was determined by incubating 500 µL aliquots of protein (1 µM concentration in 20 mM sodium acetate buffer, pH 4.6) at 30 °C, 40 °C, 55 °C, 80 °C and 100 °C for 60 min, and at 80 °C and 100 °C for 30 min. After heat treatment, the samples were allowed to cool to RT and bioassayed for toxic activity as above.

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2.3. Results

2.3.1. Expression of different forms of Ptr ToxB

All N-terminus fusion proteins (His- and GST-tagged Ptr ToxB from isolates Alg3-24 and 90-2) were successfully over-expressed in *E. coli*. However, the proteins were expressed in the insoluble fraction. Modification of culture conditions did not enhance solubility, nor did changing the length of the incubation period, increasing aeration of the cultures, or using lower concentrations of L-arabinose for the induction of expression (results not shown). Therefore, it was necessary to denature and re-fold the proteins in vitro in order to obtain active soluble forms. His-fusion proteins were expressed at 37 °C, solubilized in 8 M urea, and batch purified using Ni-NTA agarose. As GST-fusion proteins were relatively pure in the insoluble fraction, they were solublized in 8 M urea but were not purified further. Re-solubilized His- and GST-fusions were re-folded in vitro as described above and visualized by SDS-PAGE. Approximately 0.85 mg of affinity purified His-fusion protein or 16 mg of partially pure GST-fusion protein were recovered into the soluble fraction from a 160 mL volume of culture.

As His-fusions consisted of a TEV protease recognition site (Parks *et al.*, 1995) separating the N-terminal His-tag from the Ptr ToxB ORF (including an 11 aa portion of the signal peptide for Alg3-24 and an 8 aa portion for 90-2), protein products 11.1 and 10.9 kDa in size were expected for Alg3-24 and 90-2, respectively. Corresponding

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bands of approximately 11 kDa mass were detected by SDS-PAGE (Fig. 2), and after digestion with rTEV protease, two bands were obtained, approximately 7.5 kDa and 3.5 kDa in size, corresponding to Ptr ToxB (with a portion of the signal peptide) and the cleaved His-tag, respectively (Fig. 2). A third band with a mass of 29 kDa corresponded to the rTEV protease enzyme. Similarly, GST-tagged Ptr ToxB proteins from Alg3-24 and 90-2 were expected to be 36.2 and 36.0 kDa in mass, respectively, and bands of the expected size were obtained (approximately 36 kDa, Fig. 3). The GST-tag was also successfully removed from the proteins by treatment with rTEV protease, and after cleavage, two bands, 28.5 and 7.5 kDa in size, were observed, corresponding to the cleaved GST-tag and the toxin protein, respectively (Fig. 3). As was the case with native Ptr ToxB purified from fungal culture filtrates (Strelkov *et al.*, 1999), the heterologously expressed forms of the toxin migrated ahead of the 6.5 kDa molecular mass marker during SDS-PAGE (Fig. 2 and Fig. 3). However, so did native Ptr ToxB included as a control (Fig. 2, lane 2).

2.3.2. Activity of toxin proteins

The activity of the different forms of the toxin was tested by infiltration into the leaves of Ptr ToxB-sensitive and insensitive wheat genotypes, as described in the materials and methods. Activity did not differ in His-tagged or GST-tagged Ptr ToxB from isolate Alg3-24, nor did it differ after removal of the respective tags. At concentrations greater than 0.2 μM, Ptr ToxB from Alg3-24 induced chlorosis symptoms that were visible 48 h after infiltration of sensitive 'Katepwa' leaves, and

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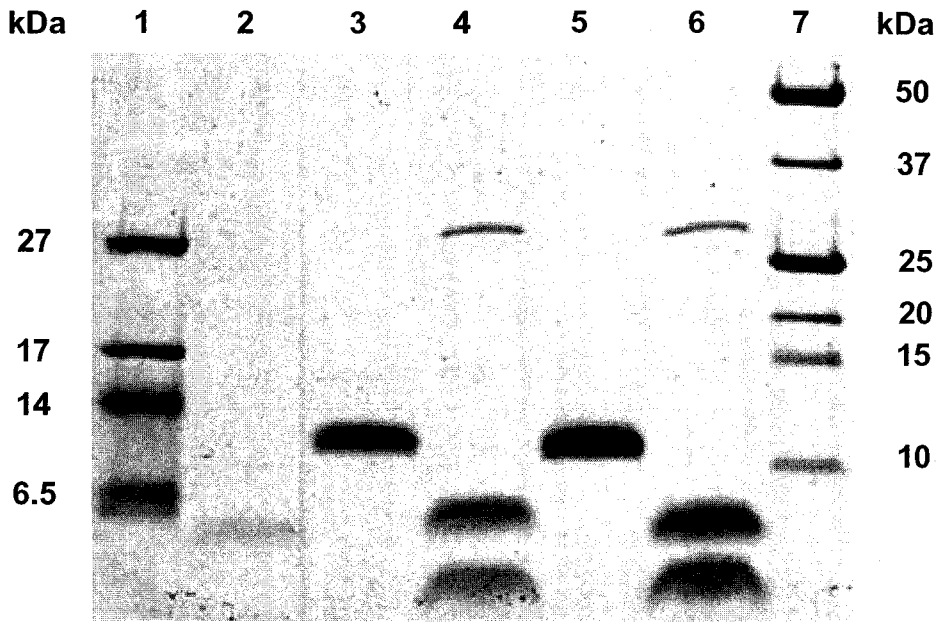

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of heterologously expressed, purified and refolded His-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*. Two µg samples of His-tagged Ptr ToxB from Alg3-24 or 90-2 were loaded per lane and visualized by staining with 0.05% Coomassie blue, after electrophoresis in a 10% Tris-tricine separating gel. Lane 1: low molecular mass markers (10 µg total protein; Polypeptide SDS-PAGE Molecular Weight Standards, Bio-Rad), lane 2: native Ptr ToxB purified from culture filtrates of Alg3-24 (1 µg total protein), lane 3: His-tagged Ptr ToxB from Alg3-24, lane 4: Ptr ToxB from Alg3-24 (middle band) after digestion with rTEV protease (top band), lane 5: His-tagged Ptr ToxB from 90-2, lane 6: Ptr ToxB from 90-2 (middle band) after digestion with rTEV protease (top band), and lane 7: molecular mass markers (5.4 µg total protein; Precision Plus Protein Standards, Bio-Rad). The cleaved His-tag is visible as the bottom band in lanes 4 and 6.

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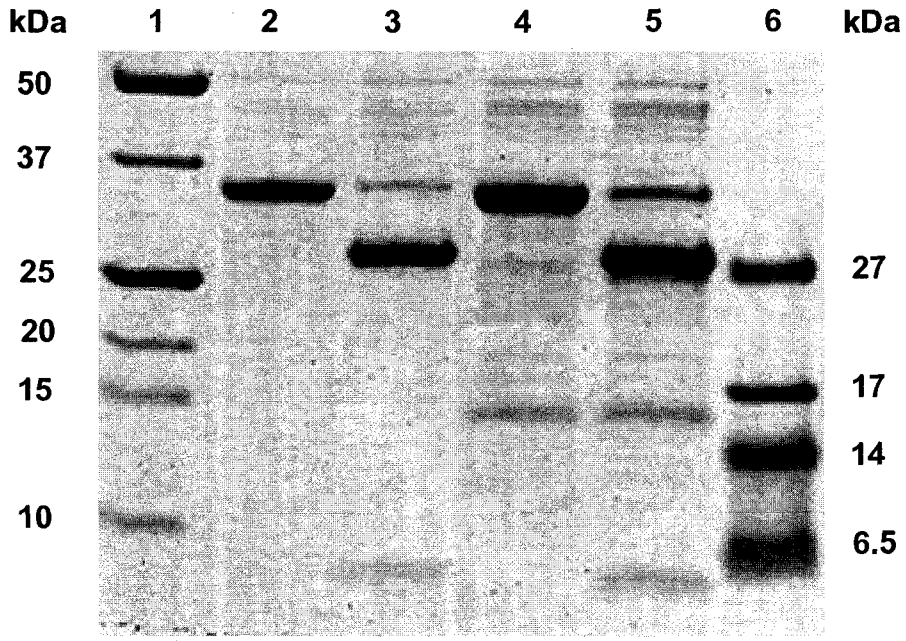

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of heterologously expressed and refolded GST-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*. Five µg samples of GST-tagged Ptr ToxB from Alg3-24 and 10 µg of GST-tagged Ptr ToxB from 90-2 were loaded per lane and visualized by staining with 0.05% Coomassie blue, after electrophoresis in a 10% Tris-tricine separating gel. Lane 1: molecular mass markers (5.4 µg total protein; Precision Plus Protein Standards, Bio-Rad), lane 2: GST-tagged Ptr ToxB from Alg3-24 (strong band at approximately 36 kDa), lane 3: Ptr ToxB from Alg3-24 (bottom band) after digestion with rTEV protease, lane 4: GST-tagged Ptr ToxB from 90-2 (strong band at approximately 36 kDa), lane 5: Ptr ToxB from 90-2 (bottom band) after digestion with rTEV protease, and lane 6: low molecular mass markers (10 µg total protein; Polypeptide SDS-PAGE Molecular Weight Standards, Bio-Rad). The cleaved GST-tag is visible as the strong band at approximately 28.5 kDa in lanes 3 and 5.

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which became very strong by 72 h. By 96 h after infiltration, some necrosis was observed at the center of the infiltrated area (Fig. 4). At toxin concentrations of 0.1 μM or less, chlorosis was not observed until 72 h after treatment, with only very weak symptoms observed at 10 nM and no symptoms detectable at a 1 nM concentration (Table 2). No chlorosis developed after infiltration of the toxin-insensitive cultivar Glenlea with Ptr ToxB, regardless of the concentration used (Fig. 4). Similarly, no chlorosis was detected after infiltration of either 'Katepwa' or 'Glenlea' with buffer-only controls.

The activity of His- and GST-tagged Ptr ToxB from isolate 90-2, as well as its activity after removal of the tags, was also tested. It appeared that all forms of the recombinant toxin from this isolate possessed little or no chlorosis-inducing activity. On the sensitive cultivar Katepwa, only trace levels of localized chlorosis were observed, beginning at 96 h after treatment. These weak symptoms developed only when the toxin was applied at concentrations greater than 0.1 μM (Fig. 5). At lower concentrations, no symptoms were detectable at any time. Similarly, no symptoms developed after infiltration of the toxin-insensitive 'Glenlea' with any concentration of Ptr ToxB derived from 90-2 (Table 2). The buffer used (50 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, or 20 mM sodium acetate, pH 4.6) did not influence activity of any form of the toxin, and therefore only the results using 20 mM sodium acetate (pH 4.6) are presented.

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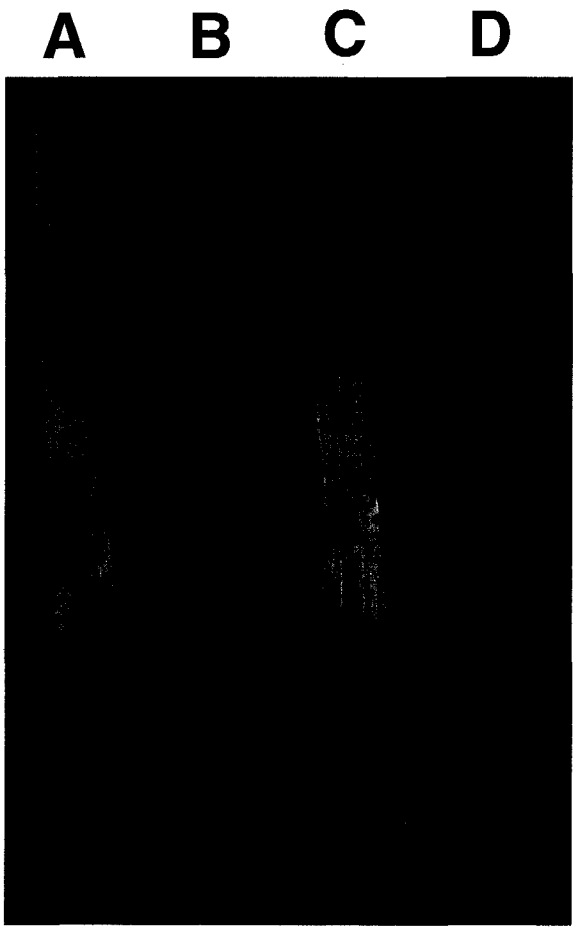

Figure 4. Symptoms induced by His- and GST-tagged Ptr ToxB from isolate Alg3-24 of *Pyrenophora tritici-repentis*. Seedlings of the Ptr ToxB-sensitive wheat cv. Katepwa and the insensitive cv. Glenlea were infiltrated at the 3 to 4 leaf stage with 1 μM solutions of the toxin (in 20 mM sodium acetate, pH 4.6), and were photographed 6 days after treatment. (A) 'Katepwa' leaf infiltrated with His-tagged toxin, (B) 'Glenlea' leaf infiltrated with His-tagged toxin, (C) 'Katepwa' leaf infiltrated with GST-tagged toxin, and (D) 'Glenlea' leaf infiltrated with GST-tagged toxin. Neither cultivar developed any symptoms after infiltration with buffer-only controls (not shown).

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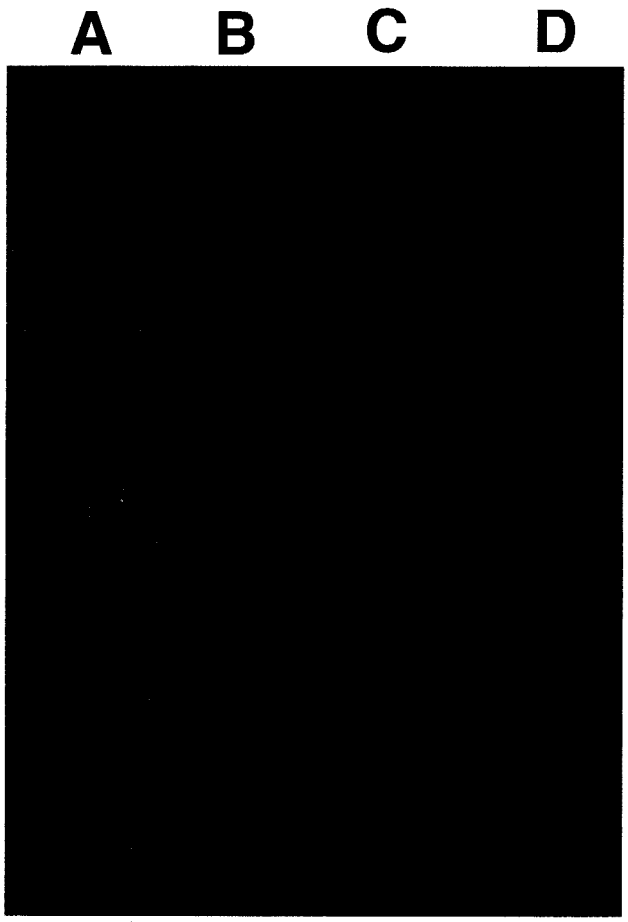

Figure 5. Symptoms induced by His- and GST-tagged Ptr ToxB from isolate 90-2 of *Pyrenophora tritici-repentis*. Seedlings of the Ptr ToxB-sensitive wheat cv. Katepwa and the insensitive cv. Glenlea were infiltrated at the 3 to 4 leaf stage with 1 μM solutions of the toxin (in 20 mM sodium acetate, pH 4.6), and were photographed 6 days after treatment. (A) 'Katepwa' leaf infiltrated with His-tagged toxin, (B) 'Glenlea' leaf infiltrated with His-tagged toxin, (C) 'Katepwa' leaf infiltrated with GST-tagged toxin, and (D) 'Glenlea' leaf infiltrated with GST-tagged toxin. Neither cultivar developed any symptoms after infiltration with buffer-only controls (not shown).

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Table 2. Induction of chlorosis by His- and GST-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*.

Concentration *	**Isolate Alg3-24**		**Isolate 90-2**	
	His-fusion (pH 4.6)	GST-fusion (pH 4.6)	His-fusion (pH 4.6)	GST-fusion (pH 4.6)
2.0 µM	+ +	+ +	trace	trace
1.0 µM	+ +	+ +	trace	trace
0.5 µM	+ +	+ +	trace	trace
0.2 µM	+ +	+ +	trace	trace
0.1 µM	+	+	trace	trace
10 nM	+/– (localized)	+/– (localized)	–	–
1 nM	–	–	–	–

*Toxin solutions were infiltrated into the leaves of the susceptible wheat cv. Katepwa at the concentrations indicated, and scored for symptom development 6 days later. Reactions indicated are severe chlorosis (++), visible chlorosis (+), low to moderate chlorosis (+/–) and absence of chlorosis (–). Trace levels of chlorosis were very weak and localized to the center of the infiltration site.

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2.3.3. Effect on pigment concentrations

The total chlorophyll concentration in toxin-sensitive 'Katepwa' seedlings infiltrated with His-tagged Ptr ToxB from isolate Alg3-24 begun to decline 48 h after treatment, dropping from 1.89 mg/g tissue at 0 h to 0.84 mg/g tissue at 120 h (Fig. 6). A much smaller decrease was also observed beginning at 96 h after treatment with the His-tagged Ptr ToxB from isolate 90-2, with the total chlorophyll concentration decreasing from 1.88 mg/g tissue at 0 h to 1.61 mg/g tissue at 120 h (Fig. 6). The rates of decline were similar in chlorophyll a and b, and the ratio between the pigments remained constant (results not shown). No decline was observed in the toxin-insensitive cultivar Glenlea after treatment with either form of the toxin, and the total chlorophyll concentration remained in the 1.74 to 2.15 mg/g tissue range throughout the time course of the study.

Patterns similar to those observed for chlorophyll were also observed with respect to carotenoid concentrations. After infiltration of 'Katepwa' tissue with His-tagged Ptr ToxB from Alg3-24, total carotenoid concentration dropped from 0.56 to 0.38 mg/g tissue, while a much smaller decline (from 0.53 to 0.50 mg/g tissue) was observed after treatment with the form of the toxin from 90-2 (Fig. 7). No significant changes were observed with respect to carotenoid concentration after treatment of 'Glenlea' leaves with His-tagged Ptr ToxB from either isolate. Similarly, no significant changes were observed in chlorophyll or carotenoid concentrations after treatment of 'Katepwa' or 'Glenlea' tissue with buffer-only controls (results not shown).

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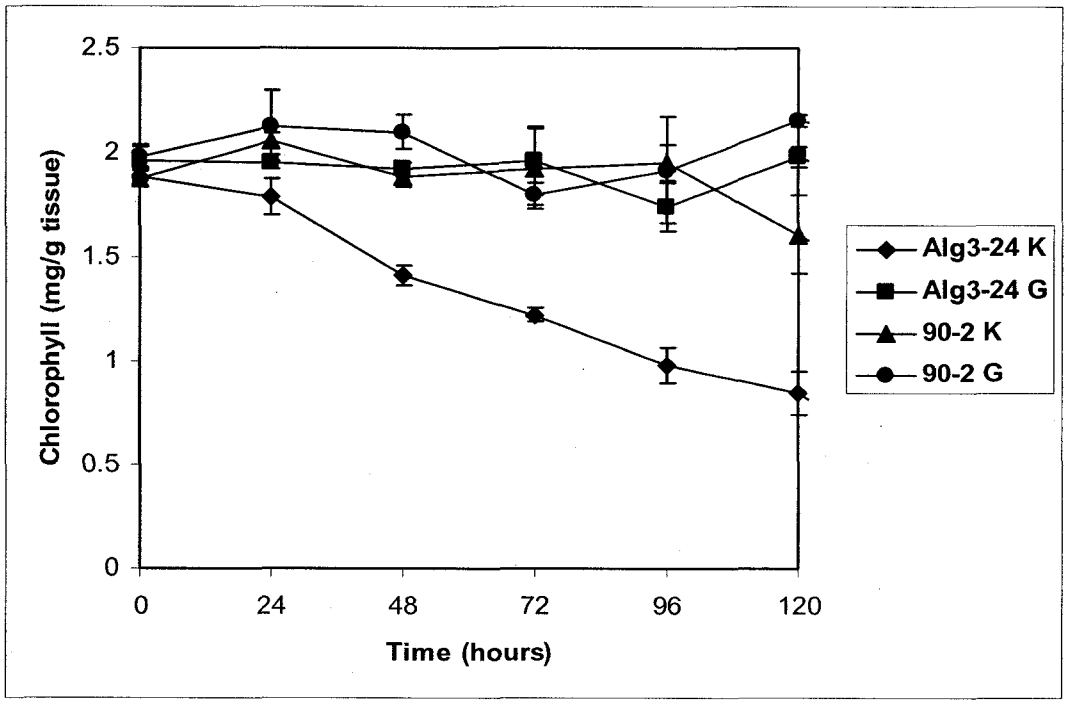

Figure 6. Total chlorophyll concentration over time after infiltration of wheat cultivars Katepwa (K; Ptr ToxB-sensitive) and Glenlea (G; Ptr ToxB-insensitive) with His-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*. Wheat leaves were infiltrated with 1 μM solutions of toxin, pigments were extracted in 80% acetone (v/v) and concentrations were determined using the equations of Lichtenhaler (1987). Treatment of 'Katepwa' and 'Glenlea' tissue with buffer-only controls did not significantly alter the chlorophyll concentration (not shown). Error bars indicate the standard error.

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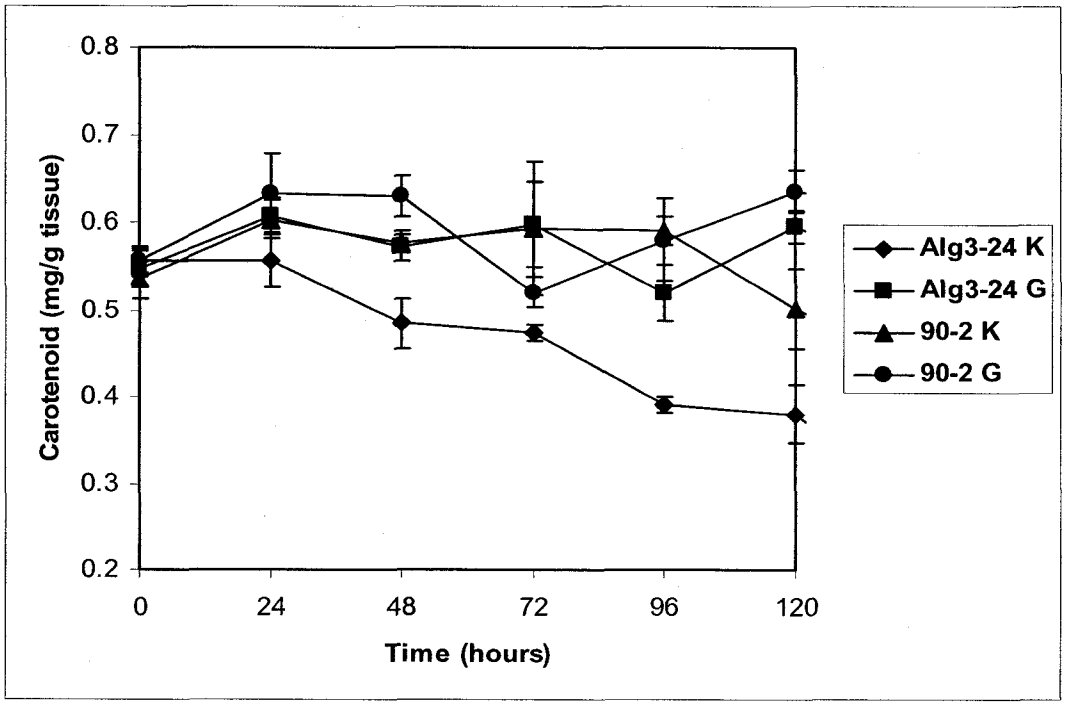

Figure 7. Total carotenoid concentration over time after infiltration of wheat cultivars Katepwa (K; Ptr ToxB-sensitive) and Glenlea (G; Ptr ToxB-insensitive) with His-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*. Wheat leaves were infiltrated with 1 μM solutions of toxin, pigments were extracted in 80% acetone (v/v) and concentrations were determined using the equations of Lichtenhaler (1987). Treatment of 'Katepwa' and 'Glenlea' tissue with buffer-only controls did not significantly alter the carotenoid concentration (not shown). Error bars indicate the standard error.

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2.3.4. Heat stability

The thermal stability of 1 μM concentrations of His-tagged Ptr ToxB from isolates Alg3-24 and 90-2 was evaluated by subjecting the proteins to various heat-period treatments and then testing for toxin activity via a bioassay. Strong chlorosis still developed after heating the His-tagged Ptr ToxB from Alg3-24 for 60 min at 30 °C, 40 °C or 55 °C. Somewhat weaker symptoms developed after treatment for 30 min at 80 °C, and a low level of chlorosis was observed after 60 min at 80 °C. Only trace levels of chlorosis were observed after heating the His-tagged toxin from Alg3-24 at 100 °C for 30 or 60 min. In the case of His-tagged Ptr ToxB from 90-2, trace levels of chlorosis were observed after heating for 60 min at 30 °C, 40 °C and 55 °C, but all activity was lost if the toxin was heated at higher temperatures. The heat stability of the His-tagged Ptr ToxB proteins is summarized in Table 3.

2.4. Discussion

To characterize the chlorosis-inducing activity of the different forms of Ptr ToxB in toxin-sensitive and insensitive wheat genotypes, it was necessary to heterologously express the protein, since purification of Ptr ToxB from fungal cultures is laborious and time consuming. Furthermore, it is difficult to purify the form of the toxin produced by avirulent isolates, since there is no simple method to track toxin activity during the purification protocol, and protein expression has not been confirmed (Strelkov, 2002). Active wild-type Ptr ToxB from virulent race 5 isolate Alg3-24 and the form of the toxin encoded by the *ToxB* homolog in avirulent race 4

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Table 3. Heat stability of His-tagged Ptr ToxB from isolates Alg3-24 and 90-2 of *Pyrenophora tritici-repentis*.

Treatment (temperature/time)*		**Isolate Alg3-24**	**Isolate 90-2**
30 °C	60 min	++	trace
40 °C	60 min	++	trace
55 °C	60 min	++	trace
80 °C	30 min	+	–
	60 min	+/–	–
100 °C	30 min	trace	–
	60 min	trace	–

*Toxin solutions (1 μM) were infiltrated into the leaves of the susceptible wheat cv. Katepwa after heating at different temperatures for varying lengths of time. Leaves were scored for symptom development 6 days later. Reactions indicated are severe chlorosis (++), visible chlorosis (+), low to moderate chlorosis (+/–) and absence of chlorosis (–). Trace levels of chlorosis were very weak and localized to the center of the infiltration site.

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isolate 90-2 were successfully expressed as His- and GST-tagged N-terminal fusion proteins in *E. coli*. The specific activity and symptoms induced by heterologously expressed His- or GST-tagged Ptr ToxB from isolate Alg3-24 were very similar to those observed when wheat leaves were treated with native toxin purified from culture filtrates of the same isolate (Strelkov *et al.*, 1999). The recombinant toxin caused strong chlorosis when applied at concentrations of 0.1 μM or greater, and could induce weak symptoms at concentrations as low as 10 nM (Table 2), which is similar to the minimum concentration reported for activity of native Ptr ToxB (Strelkov *et al.*, 1999). In addition, the onset of symptom development (beginning at 48 h after treatment) was consistent with that observed after infiltration of sensitive tissue with the native toxin (Strelkov *et al.*, 1998). At higher concentrations, native (Strelkov *et al.*, 1999) and heterologously expressed Ptr ToxB (Fig. 4) also induced limited necrosis at the center of the infiltration site. Although the effect of the GST-tagged Ptr ToxB on chlorophyll and carotenoid concentrations was not measured, the declines induced by the His-tagged protein (Figs. 6 and 7) were of a similar magnitude and pattern to those induced by the native toxin (Strelkov *et al.*, 1998). Similar levels of toxin activity were also observed after digestion of the fusion proteins with rTEV protease, suggesting that the His- and GST-moieties did not influence toxin action. This is particularly interesting with respect to the GST-tag, which has a molecular mass approximately 4× greater than that of the mature native toxin. Apparently, the presence of a His- or GST-tag and TEV protease recognition site adjacent to the N-terminal domain of Ptr ToxB does not interfere with its interaction with a putative host target or receptor. Similarly, inclusion

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of portions of the signal peptide in the heterologously expressed proteins did not appear to influence chlorosis-inducing activity.

In contrast to the His- or GST-tagged Ptr ToxB from Alg3-24, the form of the toxin expressed from avirulent isolate 90-2 possessed only trace levels of activity (Fig. 5). Given that the N-terminal tags did not influence the specific activity of the wild-type toxin, it is unlikely that the low activity observed for Ptr ToxB from 90-2 was a consequence of the expression system used. More likely, it may have resulted from the differences that exist between the two forms of the protein. Comparison of the *ToxB* ORF from isolates Alg3-24 and 90-2 indicates the presence of 13 aa substitutions and 1 insertion in the predicted protein product from 90-2 (Strelkov and Lamari, 2003). Although a functional or active site has not been demonstrated for Ptr ToxB action, it is possible that one or more of these aa changes are situated on such a site, resulting in a loss of activity. For instance, modification of an RGD motif in Ptr ToxA resulted in the loss of its necrosis-inducing activity (Meinhardt *et al.*, 2002). It is also possible that the sequence differences between the two forms of Ptr ToxB result in structural changes in the proteins, which in turn affect toxin activity. However, analysis using the program PSIRED (Jones, 1999) predicted no differences in the secondary structures of Ptr ToxB from isolates Alg3-24 and 90-2. The protein sequences were also analyzed using several programs for predicting tertiary structures (Lambert *et al.*, 2002; Lund *et al.*, 2002), but no significant results were obtained. Therefore, the effect of the aa changes on protein structure and/or any putative functional site will have to be

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empirically examined, by the resolution of the crystal structure and site-directed mutagenesis of wild-type Ptr ToxB.

There are a number of potential sites for post-translational modifications on both forms of the mature toxin protein, including two phosphorylation sites (Ser-46 and Tyr-35) on Ptr ToxB from Alg3-24, and three phosphorylation sites (Ser-31, Ser-52 and Tyr-35) on Ptr ToxB from 90-2, which were recognized using the NetPhos 2.0 program (Blom *et al.*,1999). However, the expression of active Ptr ToxB (at least from Alg3-24) in *E. coli* suggests that post-translational modifications are not necessary for toxin action. Nevertheless, while the predicted molecular mass for mature Ptr ToxB from Alg3-24 is 6,542 Da, mass spectral analysis of native toxin purified from culture filtrates indicated a mass of 6,612 Da (± 7) (Strelkov *et al.*,1999). Since phosphorylation results in a mass increase of 80 Da, it is possible that at least one of the aa residues might be phosphorylated in wild-type Ptr ToxB, resulting in a mass of 6,622 Da, which is very close to the upper range of the value obtained by mass spectrometry. The significance of such a modification, if it occurs, is unclear, since it is not required for chlorosis-inducing activity.

We have previously shown that the form of *ToxB* found in 90-2 is an active gene, which is transcribed at low levels in conidia of the fungus (Strelkov *et al.*, 2006). On-going research indicates that this gene is transcribed in culture and in planta as well, albeit also in very small quantities (S. Amaike and S.E. Strelkov, unpublished data). Regardless of the level of *ToxB* gene expression in race 4 isolates such as 90-2, the low level of activity of the toxin protein that it encodes may be sufficient to explain

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the avirulent nature of these isolates, at least on the hosts of the tan spot differential set. Nevertheless, the occurrence of identical *ToxB* homologs in race 4 isolates from locations that are geographically distant (Strelkov and Lamari, 2003; Martinez *et al.*, 2004) suggests that this form of the gene may play some role in their biology, as it is unlikely that avirulent isolates of *P. tritici-repentis* would maintain identical forms of *ToxB* unless there was selection pressure to do so (Strelkov *et al.*, 2006). Since the Ptr toxins, like most other host-specific toxins, do not control vital biological functions in the pathogen (Strelkov and Lamari, 2003), this role might be related to pathogenicity, at least in non-wheat hosts (Martinez *et al.*, 2004). Indeed, isolates of *P. tritici-repentis* recovered from non-cereal grasses were found to be predominantly race 4 (Ali and Francl, 2003). Further studies are required to determine the function, if any, of the form of Ptr ToxB in avirulent race 4 isolates. The availability of large quantities of heterologously expressed toxin, both of the wild-type and from 90-2, will facilitate this and other research into the role of Ptr ToxB in disease development and virulence.

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2.5. References

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3. Ptr ToxB-induced changes in the leaf proteome of toxin-sensitive wheat (*Triticum aestivum* L.)

3.1. Introduction

Tan spot, caused by the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), is an important foliar disease of hexaploid and durum wheat. The disease occurs throughout the major wheat growing regions of the world and causes yield losses of up to 50% (Hosford, 1982; De Wolf *et al.*, 1998). As *P. tritici-repentis* is a stubble-borne pathogen, the incidence of tan spot has increased since the 1970's, as a result of a shift by farmers toward soil conservation practices, such as zero and minimum tillage, which retain stubble on the soil surface (Hosford, 1982; Bailey, 1996). Tan spot is associated with the development of two characteristic symptoms on infected leaves: tan necrosis and extensive chlorosis (Lamari and Bernier, 1989a; Lamari and Bernier, 1989b). These symptoms often occur as oval-shaped necrotic lesions that are surrounded by a chlorotic border or halo (Wiese, 1987). The chlorosis and necrosis symptoms are induced by the action of at least three host-selective toxins (HSTs), which are differentially produced by eight known races of *P. tritici-repentis* (Lamari and Bernier, 1989b; Lamari *et al.*, 1995; Strelkov *et al.*, 2002; Lamari *et al.*, 2003). Ptr ToxA, encoded by the *ToxA* gene, is a 13.2 kDa proteinaceous toxin that causes necrosis on susceptible wheat lines/cultivars (Ballance *et al.*, 1989; Tuori *et al.*, 1995; Ciuffetti *et al.*, 1998). A second HST, Ptr ToxB, induces chlorosis and is also a protein,

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6.61 kDa in size (Orolaza *et al.*, 1995; Strelkov *et al.*, 1999). Ptr ToxB is encoded by the multiple copy gene *ToxB*, clones of which have been obtained by several independent research groups (Martinez *et al.*, 2001; Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). The least characterized of the HSTs produced by *P. tritici-repentis* is Ptr ToxC, which also induces chlorosis, but on different wheat genotypes than Ptr ToxB. Unlike Ptr ToxA and Ptr ToxB, Ptr ToxC appears to be a low molecular mass, polar, non-ionic compound (Effertz *et al.*, 2002).

Ptr ToxB has been hypothesized to induce chlorosis by disrupting the normal photosynthetic mechanisms of the host, leading to chlorophyll photooxidation (Strelkov *et al.*, 1998). However, the mode of action of this toxin is not fully understood. In an effort to gain a better understanding of the mechanisms involved in Ptr ToxB action and the development of chlorosis, we undertook an analysis of toxin-induced changes in the proteome of the Ptr ToxB-sensitive wheat cultivar Katepwa. Proteomics combined with two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) are powerful tools for understanding the changes induced in plant hosts in response to biotic or abiotic stresses (Kav *et al.*, 2004; Yajima *et al.*, 2004; Sharma *et al.*, 2007). Using these techniques, specific proteins differentially induced by pathogen challenge, and which may play critical roles in mediating host-pathogen interactions and host resistance and/or defense mechanisms, can be reproducibly visualized and identified. Although there have been several reports examining changes in the wheat proteome induced by pathogens such as *Fusarium graminearum* (Fusarium head blight) and *Puccinia triticina* (leaf rust) (Rampitsch *et al.*, 2006; Wang

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et al., 2005; Zhou *et al.*, 2006), to our knowledge none have examined the interaction between wheat and the chlorosis-inducing isolates of *P. tritici-repentis*. The production, by the fungus, of host-specific toxins that serve as pathogenicity factors makes tan spot an ideal system to analyze via a proteomics approach, since the effect of the toxins on the host can be studied directly, in the absence of any non-specific changes that may be induced by fungal inoculation or infection.

3.2. Materials and methods

3.2.1. Plant material

The wheat cultivar Katepwa (sensitive to Ptr ToxB) was used throughout this study. Plants were grown in plastic pots (12.7 cm in diameter) filled with Metro-Mix® 220 soil (Grace Horticultural products, Ajax, ON) at a density of 5 seedlings per pot. The plants were maintained in a growth cabinet at 21/18 °C (day/night) with a 16-h photoperiod at a light intensity of 180 μmol photons m^{-2} s^{-1}. Seedlings were grown to the 3-4 leaf stage and watered and fertilized as required.

3.2.2. Production of Ptr ToxB and treatment of host leaves

Hexahistidine (His)-tagged Ptr ToxB from race 5 isolate Alg3-24 of *P. tritici-repentis* was heterologously expressed in *Escherichia coli* and purified as previously described (Chapter 2). In brief, the *ToxB* open reading frame (ORF) was amplified according to the GATEWAY™ Cloning Technology Adapter PCR protocol (Invitrogen, Carlsbad, CA), using forward primer YMK1F (5'-AA AAA GCA GGC

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TTC GAA AAC CTG TAT TTT CAG GGC GTG GCT ATC CTC CCT GCT G-3') and reverse primer YMK1R (5'-A GAA AGC TGG GTC GGA AAA CTG TGC TAT GCC AGA-3'). The amplicons were inserted between the *att*P1 and *att*P2 sites of the pDONR™ 221 vector and then transferred to the pDEST™ 17 vector for N-terminal His-fusion protein expression in BL21-AI™ *E. coli* cells, as per the manufacturer's instructions (Invitrogen). Over-expressed fusion proteins were denatured in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, 20 mM βME, 10mM imidazole, pH 8.0), and purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Mississauga, ON). Batch purified His-tagged Ptr ToxB was refolded in vitro by dialysis against 5 mM sodium acetate buffer (pH 4.6) and concentrated (4×) by freeze-drying. A 1 μM concentration of purified His-tagged recombinant protein (in 20 mM sodium acetate buffer, pH 4.6) was infiltrated into the second and third leaves of wheat seedlings at the 3-4 leaf stage, using a Hagborg (1970) device. Controls were infiltrated with buffer only.

3.2.3. Sample preparation and protein extraction

Leaf segments (total weight 350 mg), randomly chosen 24 h after infiltration with Ptr ToxB or buffer, were cut, weighed and flash frozen in liquid nitrogen. Proteins from each treatment were extracted according to a method modified from Damerval *et al.* (1986). Pooled leaf tissue was ground with a mortar and pestle to a fine powder in liquid nitrogen. The homogenate was transferred to a pre-chilled 1.5 mL microcentrifuge tube and re-suspended in 1 mL of ice-cold acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% (w/v) DTT. After vortexing for

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several seconds, samples were incubated at –20 °C for 1 h. The samples were then centrifuged at 16,000 × g for 15 min at 4 °C and the supernatants discarded. Each pellet was re-suspended in 1.2 mL of ice-cold acetone containing 0.07% (w/v) DTT, vortexed vigorously, and incubated again at –20 °C for 1 h. The samples were centrifuged as above and the wash step repeated an additional four times. The washed pellets were vacuum-dried at room temperature (RT) until the acetone evaporated completely. The dried protein pellets were dissolved in 550 µL of rehydration/sample buffer (RB) (8 M urea, 2% [w/v] CHAPS, 50 mM DTT, 0.2% [w/v] Bio-Lyte 3/10, 0.001% [w/v] Bromophenol blue) (Bio-Rad, Mississauga, ON) containing 2 mM tributylphosphine (TBP), vortexed vigorously, and incubated overnight at 4 °C. In the final step, the samples were centrifuged as above and the supernatants transferred to clean tubes and stored at –20 °C. Protein concentrations were determined according to the method of Bradford (1976), using the Protein Assay Dye Reagent (Bio-Rad), with bovine serum albumin (Pierce Biotechnology Inc., Rockford, IL) included as a standard. As this was a preliminary study, the results presented are from three technical replicates of one biological repetition of the experiment.

3.2.4. Two-dimensional electrophoresis

Five hundred µg of each protein extract, in a total volume of 300 µL RB containing 2 mM TBP, were passively rehydrated overnight into immobilized pH gradient (IPG) strips (17 cm, pH 4-7, Bio-Rad) as per the manufacturer's instructions. Rehydrated IPG strips were then transferred into a focusing tray, covered with mineral oil, and subjected to isoelectric focusing (IEF), which was conducted in a

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PROTEAN® IEF Cell (Bio-Rad) using the manufacturer's pre-set 4-step protocol: (1) 250 V was applied for 15 minutes to remove salt ions and charged impurities, (2) the voltage reached a maximum of 10,000V with a linear increase over 3 h, (3) focusing was conducted at a constant 10,000V for 60,000 volt-hours with a 50 μA limit/IPG strip, and (4) a 500 V hold step was maintained until the run was stopped, in order to prevent over-focusing or protein drift.

After IEF, the IPG strips were removed from the focusing tray and equilibrated 2× in 5 mL equilibration buffer 1 [6M urea, 2% (w/v) sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl, pH 8.8, 20% (v/v) glycerol and 130 mM DTT] for 15 min at RT with gentle shaking, to reduce sulfhydryl groups. Following saturation with equilibration buffer 1, the strips were incubated 2× in equilibration buffer 2 [6M urea, 2% (w/v) SDS, 0.375 M Tris-HCl, pH 8.8, 20% (v/v) glycerol and 135 mM iodoacetamide] for 15 min at RT with gentle agitation, to alkylate the reduced sulfhydryl groups. For separation in the second dimension, polyacrylamide gel electrophoresis (PAGE) was carried out in a PROTEAN® II xi Cell (Bio-Rad) under denaturing conditions with SDS. This was run using the buffer system of Laemmli (1970) on 13% polyacrylamide gels at a constant voltage (90V). Gels were stained with Coomassie blue using a Colloidal Blue Staining Kit (Invitrogen) according to the manufacturer's instructions (200 mL of staining solution/gel), then destained overnight in distilled water.

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3.2.5. Image analysis

Stained 2-D gels were scanned on a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed using PDQuest software (Bio-Rad). Protein spots were automatically detected and matched, followed by manual validation to eliminate false, mismatched and unmatched spots. Protein spots that were significantly different between treatments were identified using the Student's *t*-test feature of the PDQuest software, at a significance level of 95%. The fold changes in the spot intensities between the treatments were calculated and the spots carefully excised from the gels using a sterile surgical blade for each spot. Excised spots were subjected to electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS) for protein identification as described below.

3.2.6. ESI-Q-TOF-MS/MS

Identification of excised protein spots was performed at the Institute for Biomolecular Design (IBD), University of Alberta, Edmonton, Canada, using ESI-Q-TOF MS/MS as described in Yajima and Kav (2006). Gel processing was conducted on an automated MassPREP. Station (Micromass, Manchester, UK) as per the manufacturer's instructions. Briefly, gel pieces were destained, reduced in 10 mM DTT, alkylated in 55 mM iodoacetamide, and digested with sequencing-grade modified trypsin (6 ng/μL in 25 μL of 50 mM ammonium bicarbonate, pH 8.0) at 37°C for 5 h. The tryptic peptides were extracted using (sequentially) 1% (v/v) formic acid, 2% (v/v) acetonitrile, and 50% (v/v) acetonitrile, and subjected to liquid chromatography tandem MS (LC/MS/MS) analysis on a Micromass Q-TOF-2 mass

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spectrometer (Micromass), coupled with a Waters CapLC capillary HPLC (Waters Corp., Milford, MA). The protein samples were run on a PepMap C18 column (300 micron × 5 mm; LC Packings, Sunnyvale, CA), followed by separation on a PicoFrit capillary reversed-phase column (5 μm BioBasic C18, 300 Angstrom pore size, 75μm internal diameter × 10 cm, 15 μm tip; New Objectives, Woburn, MA) using a linear water/acetonitrile gradient (0.2% [v/v] formic acid). The eluted peptides were applied to the mass spectrometer by electrospray ionization and the data generated by mass spectrometry (with a charge state of 2 or 3) used for protein identification through a search of the NCBI non-redundant database, using Mascot software (Perkins *et al.*, 1999).

3.3. Results and discussion

We hypothesized that treatment of toxin-sensitive wheat leaves with Ptr ToxB would induce proteome-level changes that would be detectable prior to the development of chlorosis. Since chlorosis develops 48 to 72 h after toxin infiltration, we chose to analyze proteome changes induced at 24 h. Analysis of a time-point occurring prior to symptom development would increase the likelihood that any changes detected could provide clues as to how chlorosis is induced, rather than being a secondary consequence of its development. Thus, these changes would have a greater chance of providing useful information as to the possible mode of action of Ptr ToxB. We infiltrated the leaves of the toxin-sensitive wheat cultivar Katepwa with buffer only or heterologously expressed, His-tagged Ptr ToxB; proteins were extracted

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from the treated tissues and separated by 2-DE, followed by analysis using specialized gel imaging software (Fig. 8). Ten protein spots were found to be significantly up-regulated in the Ptr ToxB-treated tissue, relative to the buffer controls, and were subjected to ESI-Q-TOF MS/MS (Table 4). The identities of six of these proteins were established through a database search and are summarized in Table 4.

As with most reports on the changes in protein levels induced by pathogen challenge (Wang *et al.*, 2005; Zhou *et al.*, 2005; Rampitsch *et al.*, 2006; Zhou *et al.*, 2006; Sharma *et al.*, 2007), the majority of the up-regulated proteins identified in the current study are involved in stress/defense responses, photosynthesis and/or host metabolism. Heat shock protein 70 (HSP70) like protein (spot #3), luminal binding protein (BiP; spot #2), and manganese superoxide dismutase (SOD [Mn]; spot #8) were all significantly up-regulated and are associated with stress or defense responses. HSP70 (spot #3) is amolecular chaperone and plays a role in folding of newly synthesized proteins and in maintaining protein conformation during translocation to subcellular organelles. In plants, HSP70 molecular chaperones are located in the cytosol, endoplasmic reticulum (ER), mitochondria, chloroplasts and peroxisomes (Feldman and Frydman, 2000; Sung *et al.*, 2001). However, HSP70 is also expressed in response to abiotic stresses such as heat, and confers thermotolerance to plants (Feder *et al.*, 1996). Furthermore, this protein has a fundamental role in the hypersensitive response against plant pathogens (Kanzaki *et al.*, 2003), and has been found to be induced by viruses in susceptible cells of *Arabidopsis thaliana* (Whitham *et al.*, 2003). Over-expression of HSP70 protects cells from glucose deprivation

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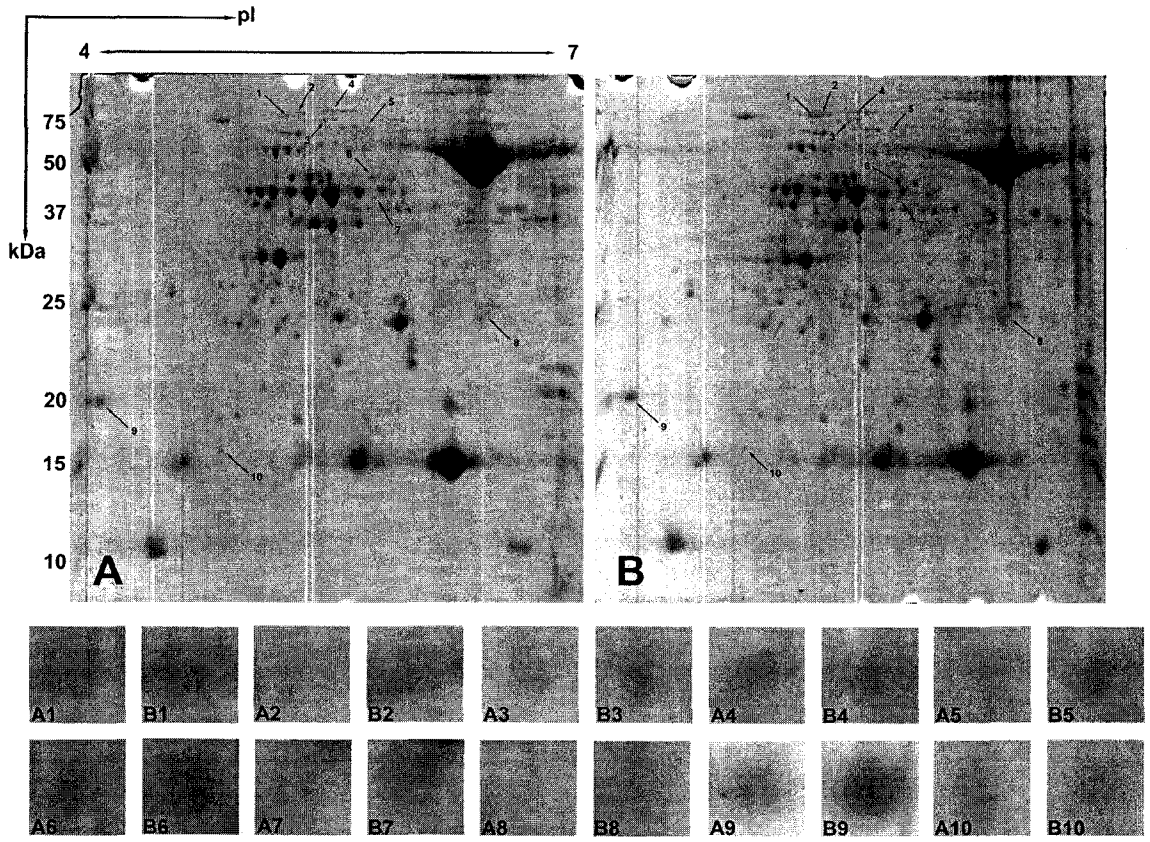

Figure 8. Colloidal coomassie blue-stained images of two-dimensional gels of wheat cv. Katepwa leaf protein extracts 24 h after treatment with (A) 20 mM sodium acetate buffer (pH 4.6) or (B) 1 μM Ptr ToxB in 20 mM sodium acetate buffer (pH 4.6). Arrows and numbers indicate protein spots that were found to be significantly different between the treatments as per the Student's *t*-test, at a significance level of 95%. Individual spots are enlarged and shown below the corresponding gel images.

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Table 4. Summary of the proteins identified by MS/MS that were found to be differentially expressed by the wheat cultivar Katepwa 24 h after treatment with Ptr ToxB.

Spot No.	MS/MS (ESI-Q-TOF)			Identity	M_r/pI	Accession No.	Status[b]
	PM/%[a]	Sequence	Score				
1	—	—	—	Not identified	—	—	2.06 ± 0.40 ↑
2	4%	FDLTGVPPAPR LSQEEIDR EAEEFAEEDKK	63	luminal binding protein (BiP) [*Arabidopsis thaliana*]	73732/5.08	gi|1303695	2.85 ± 0.28 ↑
3	1%	RFDDPQTQK	33	heat shock protein 70 like protein [*Arabidopsis thaliana*]	71415/5.31	gi|7270774	1.37 ± 0.11 ↑
4	—	—	—	Not identified	—	—	3.13 ± 0.69 ↑
5	6%	TSGEYLVK FQNALEAVK	55	phosphoglycerate mutase [*Triticum aestivum*]	29615/5.43	gi|32400802	3.47 ± 0.49 ↑
6	8%	VACETVTK NIGFISDDVGLDADR TAAYGHFGR	104	putative AdoMet synthase 3 [*Hordeum vulgare* subsp. *vulgare*]	43138/5.51	gi|68655446	2.55 ± 0.73 ↑
7	7%	YDSMLGTFK IVDNETISVDGK AVSLVLPQLK	146	glyceraldehyde 3-phosphate dehydrogenase B subunit [*Arabidopsis thaliana*]	43168/5.60	gi|336390	4.32 ± 2.58 ↑
8	10%	NLKPISEGGGEPPHGK YAGEEYEK	81	manganese superoxide dismutase [*Triticum aestivum*]	25283/7.89	gi|1654387	6.41 ± 3.39 ↑
9	—	—	—	Not identified	—	—	2.98 ± 0.96 ↑
10	—	—	—	Not identified	—	—	2.25 ± 0.30 ↑

[a] Number of peptides matched/ sequence percentage coverage

[b] Fold increase (↑) in expression of different proteins in Ptr ToxB-treated leaf tissue. Average fold changes were calculated from three technical replicates of one biological replicate. Standard errors of the mean fold changes are included.

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(Papadopoulos *et al.*, 1996), and perhaps most significantly in the context of Ptr ToxB-induced chlorosis, against the damaging effects of hydrogen peroxide (H_2O_2) (Echave *et al.*, 2002). BiP (spot #2) also belongs to the HSP70 family, and has similar functions in protein folding and translocation. Like HSP70, BiP is also a stress-induced protein (Denecke *et al.*, 1991; Kalinski *et al.*, 1995), and may confer tolerance to endogenous oxidative stress (Alvim *et al.*, 2001). In plants, SOD (spot #8) converts superoxide radicals to H_2O_2, which can be subsequently reduced to water by ascorbate peroxidase, glutathione peroxidase or catalase (Apel and Hirt, 2004). Therefore, SOD also plays an important role in helping to protect cells from oxidative stress (Alscher *et al.*, 2002; Halliwell and Gutteridge, 2000).

The identification of HSP70, BiP and SOD, all of which have been implicated in the plant response to oxidative stress, as significantly up-regulated in toxin-treated tissue seems consistent with the proposed mode of action for Ptr ToxB. The toxin was hypothesized to induce chlorosis via the photochemical bleaching of chlorophyll molecules, through the action of reactive oxygen species (ROS), as a result of a direct or indirect inhibition of photosynthesis (Strelkov *et al.*, 1998). However, up-regulation of these proteins as part of a more general stress response, or as part of an oxidative burst triggered by the presence of Ptr ToxB, cannot be ruled out. For instance, Zhou *et al.* (2005) found that SOD was up-regulated after inoculation of wheat with *F. graminearum*, indicating an oxidative burst consisting of H_2O_2 and superoxide inside pathogen-infected tissues. Examination of proteome-level changes induced by the toxin in a Ptr ToxB-insensitive wheat genotype may help to clarify the significance of

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the current results. Nevertheless, in light of previous evidence, the preliminary proteomics data support a role for ROS in Ptr ToxB-induced chlorosis.

In addition to proteins involved in stress and/or defense responses, two proteins involved in primary metabolic pathways were also found to be up-regulated 24 h after treatment of sensitive wheat leaves with Ptr ToxB, namely phosphoglycerate mutase (PGM; spot #5) and putative AdoMet synthase 3 (spot #6). Phosphoglycerate mutase is an enzyme involved in glycolysis and gluconeogenesis (carbohydrate metabolism) and has a specific role inter-converting a phosphate group between the 2- and 3- positions of a glycerate molecule (Grisolia and Joyce, 1959). The form of the enzyme found in plants is phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent (PGAMi) (Donnelly *et al.*, 2005; Grisolia and Joyce, 1959). The MS/MS data indicate a mass of 29.6 kDa for this protein (Table 4), yet the corresponding spot on the 2-D gel appears to be approximately 60-70 kDa is size (Fig. 8). Previous reports suggest a mass of 65 kDa (Bryna *et al.*, 2005) for PGAMi, which corresponds to our results with 2-D gel electrophoresis. The pI was consistent in both the MS/MS and electrophoresis analyses.

AdoMet synthase 3 (spot #6), which is also known as *S*-adenosylmethionine synthetase (SAMS), is involved in nitrogen metabolism. Specifically, this enzyme converts L-methionine and ATP into *S*-adenosyl-L-methionine (SAM) in the activated methyl cycle (Bohnert and Jensen, 1996; Hanson and Roje, 2001). However, two isoforms of AdoMet synthase 3 (type I and type II) have also been found to be expressed under stress conditions, which may be involved in lignin or ethylene biosynthesis (Broekaert *et al.*, 2006; Sanchez-Aguayo *et al.*, 2004; Schroder *et al.*,

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1997). Fluctuations in the levels of metabolic enzymes such as PGM and AdoMet synthase 3 may reflect profound physiological disturbances in Ptr ToxB-treated tissue (Strelkov, 2002). Nevertheless, only two enzymes were found to be up-regulated at the time-point examined, which was 24 h prior to the development of detectable chlorosis. Hence, ROS-mediated damage was not necessarily widespread at that time. Interference by the toxin with enzymes involved in nitrogen and carbon metabolism may also play a role in helping to re-direct energy reserves towards *P. tritici-repentis* in compatible interactions, an idea that would need to be investigated further. In a study of the compatible interaction between *Fusarium graminearum* and its wheat host, Zhou *et al.* (2006) hypothesized that an observed up-regulation of proteins associated with carbon metabolism could help the pathogen acquire carbon from the host plant.

Given the effect of Ptr ToxB on chlorophyll concentration, and the fact that it may, directly or indirectly, inhibit photosynthesis in affected leaves (Strelkov *et al.*, 1998), we were particularly interested in detecting changes in photosynthesis-related proteins. However, only one of the spots identified as up-regulated corresponded to an enzyme involved in photosynthesis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) B subunit (spot #7). This enzyme exists as two forms in higher plants: (1) a heterotetramer of two A subunits and two B subunits (A_2B_2), and (2) a homotetramer of four A subunits (A_4) (Cerff, 1979). The B subunit of the chloroplast GAPDH isozyme is encoded by the light-regulated gene *GAPB* (Chan *et al.*, 2001), and catalyzes the reversible reduction and dephosphorylation of 1,3-biphosphoglycerate to glyceraldehyde-3-phosphate in the Calvin cycle (Cerff, 1982). Therefore, the

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identification of the GAPDH B subunit as an up-regulated protein 24 h after treatment with Ptr ToxB may reflect an effect, direct or indirect, of the toxin on the carbon reduction reactions of photosynthesis. However, the failure to detect changes in any other proteins associated with this pathway makes it difficult to draw meaningful conclusions. Enzyme assays will be helpful in determining whether the activity of the chloroplast fraction of GAPDH is affected.

It is also worth noting that the large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) appeared to be down-regulated approximately 1.5 fold in Ptr ToxB-treated tissue. However, as the corresponding protein spots in the 2-D gels were tightly clustered (Fig. 8), they were difficult to differentiate, and the Rubisco data was therefore excluded from the analysis. Nevertheless, the fate of Rubisco should be investigated further, since its degradation, particular prior to the development of visible symptoms, may provide clues as to the effect of Ptr ToxB on photosynthetic processes. In a study of the *F. graminearum*/wheat interaction, it was found that the large and small subunits of this enzyme were up-regulated, but exhibited reduced molecular masses, suggesting accelerated degradation of Rubisco after infection by the pathogen (Zhou *et al.*, 2006). This led the authors to suggest that photosynthesis was disrupted or somehow decreased after infection of the host.

3.4. Concluding remarks

The advent of powerful proteomics techniques, combined with genomics and molecular biology, provides many of the tools necessary to elucidate the complex

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interactions between pathogenic microorganisms and their plant hosts. In wheat, the recent characterization of the leaf proteome (Donnelly *et al.*, 2005) provides additional opportunities to understand the changes induced by pathogenic infection. In the present study, we examined changes in the proteome observed 24 h after infiltration with the host-specific toxin, Ptr ToxB. The results obtained seem to confirm the previous suggestion that chlorosis induced by this toxin results from an inhibition of photosynthesis, resulting in chlorophyll photooxidation as illuminated thylakoid membranes become unable to dissipate excitation energy (Strelkov *et al.*, 1998). Three of the proteins identified are associated with stress or defense responses in plant tissue. In particular, these proteins, especially SOD, have roles in the protection of cells against damaging ROS. The fact that they were found to be significantly up-regulated 24 h after treatment with the toxin, prior to the development of detectable chlorosis, appears to confirm that the formation of ROS precedes the development of the chlorosis symptom, and that these molecules are therefore likely responsible for its development (Strelkov *et al.*, 1998). Moreover, the finding that these proteins were up-regulated in affected tissue seems to rule out the possibility that chlorosis develops as a consequence of the failure (by the inhibition) of the enzymatic antioxidant protective systems in the leaves.

The significance of several of the other proteins found to be up-regulated was less clear. For instance, up-regulation of PGM and AdoMet 3, which are involved in carbon and nitrogen metabolism, respectively, may simply reflect general metabolic disturbances induced by Ptr ToxB, or may indicate a shift in the metabolic machinery

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of the host, which may favor *P. tritici-repentis*. Similarly, upregulation of the chloroplast GAPDH isozyme could reflect a general disruption of photosynthetic processes, or could have specific implications regarding the effect of Ptr ToxB on the Calvin cycle. However, it should be stressed that the current study was preliminary in nature. The treatments must be repeated in order to confirm the results obtained. Furthermore, these results must be validated by other means, such as quantitative real-time PCR or enzyme assays. A good target for further validation might be SOD, given that it was the most up-regulated protein identified in the study. It may also be worthwhile to compare the effect of Ptr ToxB on the proteome of a toxin-insensitive wheat genotype, such as 'Erik,' in order to see if differences are observed, particularly with respect to the expression of stress/defense related proteins such as SOD. In addition, inclusion of additional time-points in future studies may allow us to discern any trends, as well as help us identify more proteins that are differentially expressed. Nevertheless, the current study provides a foundation from which to initiate further research into the role of Ptr ToxB in the development of tan spot of wheat, and the mechanisms by which this toxin induces chlorosis.

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4. General Discussion

The tan spot pathosystem is a mirror image of the classical gene-for-gene model (Lamari *et al.*, 2003; Strelkov and Lamari, 2003). In the gene-for-gene model, incompatibility or resistance represents the unique interaction between a host and pathogen. When an avirulence gene product produced by the pathogen is recognized by a resistance gene product produced by the host, the defense responses of the latter are triggered and no disease develops. Conversely, when the avirulence and/or resistance gene product(s) are missing, there is no recognition between host and pathogen and disease occurs. In the *Pyrenophora tritici-repentis*/wheat interaction, when the toxin(s) produced by the pathogen are recognized by a receptor or target produced by the host (i.e. "susceptibility" gene product), there is disease development or a compatible interaction. If the toxin is absent or there is no receptor or target in the host, there is no recognition and no disease. Therefore, the unique interaction is susceptibility or compatibility (Lamari *et al.*, 2003; Strelkov *et al.*, 2003), and tan spot follows the toxin model (Loegering, 1978) of host-pathogen interactions.

In order to gain further insights into the role of Ptr ToxB in the pathogenicity of *P. tritici-repentis*, we over-expressed and purified functional Ptr ToxB from virulent and avirulent isolates of the fungus in *E. coli*. We demonstrated that Ptr ToxB fusion proteins from the virulent isolate Alg3-24 are fully functional and have similar activities to native Ptr ToxB purified from culture filtrates. His-tagged and native toxin induced chlorosis at concentrations of 10 nM (Table 2) and 14 nM (Strelkov *et al.*, 1999), respectively, and caused comparable declines in pigment concentration in Ptr

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ToxB sensitive wheat leaves. Furthermore, the presence of a HIS- or GST-tag did not affect the host-specificity or ability of Ptr ToxB to induce chlorosis. Therefore, it should not be necessary to cleave these tags prior to the use of recombinant toxin in future experiments.

Interestingly, the form of Ptr ToxB expressed from the avirulent isolate 90-2 possessed only very weak or trace levels of activity. Thus, it appears that the differences in amino acid sequence between wild-type toxin and the form of the toxin in 90-2 are sufficient to eliminate most of its chlorosis-inducing activity, at least on the wheat genotypes tested. Although Ptr ToxB from 90-2 exhibits a number of differences in sequence relative to the wild-type toxin found in virulent isolates, it is identical to the form of the toxin found in other avirulent isolates, including those with fairly distant geographic origins (Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). It seems unlikely that avirulent isolates would possess identical forms of Ptr ToxB unless there was selection pressure to do so (Strelkov *et al.*, 2006). However, since the toxins of *P. tritici-repentis* do not appear to have vital biological functions apart from their role in pathogenesis, this suggests that the form of Ptr ToxB found in avirulent isolates serves a function in the interaction of the fungus with other host species (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). *Pyrenophora tritici-repentis* has a broad host range, which includes not only wheat but also many other non-cereal grass species (De Wolf *et al.*, 1998). Ali and Francl (2003) conducted a survey of the pathogen on non-cereal grasses and wheat on the Great Plains of the United States, and found that while only 5% of *P. tritici-repentis* isolates found on wheat were classified

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as race 4 (i.e. avirulent), 98% of those obtained from other hosts belonged to this race. Therefore, the "avirulent" designation describes the behaviour of race 4 isolates on the wheat differentials used, but may not reflect their pathogenic ability on other grasses (S.E. Strelkov, personal communication). In this context, it is possible to envision other roles for Ptr ToxB in disease development.

A secondary objective of our research was to examine changes in the proteome of Ptr ToxB-sensitive wheat leaves induced by treatment with the toxin. It should be stressed that this was a preliminary experiment that needs to be replicated. Nevertheless, the data for the one time-point we examined (24 h after infiltration) are consistent with previous research. Strelkov *et al.* (1998) provided evidence for the involvement of ROS in the development of Ptr ToxB-induced chlorosis. Several of the proteins that we found to be up-regulated after toxin-treatment, most notably SOD, are related to the oxidative stress response in host tissue. The fact that the increase in proteins such as SOD was observed at least 24 h prior to the development of visible chlorosis, further suggests that the increase in ROS species precedes and is not a secondary effect of symptom development. Furthermore, these data suggest that the enzymatic photoprotective systems in affected tissues remain functional, at least initially, and hence chlorosis does not develop as are result of their failure (although enzyme assays of key enzymes including SOD should serve to validate the proteome results). Therefore, our findings seem to strengthen the hypothesis that Ptr ToxB (directly or indirectly) inhibits photosynthesis, resulting in photochemical bleaching of

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the chlorophyll as illuminated thylakoid membranes become incapable of dissipating excitation energy (Strelkov *et al.*, 1998).

The current work has provided a number of tools that will enable additional research into the wheat/*P. tritici-repentis* interaction. Most notably, the ability to easily express and purify large quantities of functional Ptr ToxB from *E. coli* will allow for a large complement of additional experiments, particularly into the mode and site of toxin action. We are currently raising antibodies against the toxin protein which will allow us to initiate immunolocalization studies, intended to evaluate whether the toxin is internalized in plant cells, and if so, whether it accumulates or is associated with particular organelles or sub-cellular compartments. Of particular interest is whether a difference will be observed in terms of the fate of Ptr ToxB in toxin-sensitive and insensitive cells. Furthermore, while it was not previously possible to purify the form of Ptr ToxB from the avirulent isolate 90-2, we now have large quantities available. This will enable us to easily screen large numbers of other host genotypes, especially non-cereal grasses, for any potential activity. In addition, the availability of antibodies against Ptr ToxB will allow us to compare the production of the toxin protein by various fungal isolates differing in virulence, both in vitro and in planta, by Western blotting. Research in our lab has already shown that the toxin is expressed in toxin-sensitive (susceptible) and insensitive (resistant) wheat genotypes, by virulent and low virulence isolates, as well as to a much lower extent by the avirulent isolate 90-2 (S. Amaike and S.E. Strelkov, unpublished data). However, this work was conducted at the RNA level, and it will be important to confirm protein expression.

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In addition, as noted above, the proteomic study we conducted was preliminary in nature. Most importantly, the results obtained for the 24 h time-point that we examined must be confirmed by analysis of additional replications at that time, and the up-regulation of SOD must be confirmed by enzyme assays and/or quantitative real-time PCR. Furthermore, assessment of protein profile changes at other time-points may provide more insights into the mode of action of Ptr ToxB, particularly with respect to its effect on photosynthesis and the formation of ROS species. Any additional time-points selected for analysis should be prior to chlorosis symptom development, to increase the probability that changes observed are not secondary effects of toxin action. Comparison of proteomic profiles of toxin-sensitive and insensitive wheat genotypes may also serve to elucidate the molecular basis for sensitivity. Although potential research avenues related to the work undertaken in this project have been discussed, there are of course many other possible opportunities for study that may yield just as important or informative results. Such avenues should also be pursued, and include fungal transformation experiments to further examine the role of Ptr ToxB as a pathogenicity factor, as well characterization of additional forms of the toxin gene found in other isolates of the fungus. Together, such research will provide important insights into the role of Ptr ToxB in tan spot development, but will also serve to further understanding of the wheat/*P. tritici-repentis* interaction in general.

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