University of Alberta

Studies on the Role and Mechanism of Action of Ptr ToxB from *Pyrenophora tritici-repentis*

by

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Abstract

Tan spot is an important foliar disease of wheat caused by *Pyrenophora tritici-repentis*. This fungus produces Ptr ToxB, a host-specific toxin that induces chlorosis on sensitive host genotypes. An assessment of the effects of Ptr ToxB in a toxin-sensitive wheat line revealed that photosynthesis, as measured by infra-red gas analysis, declined significantly prior to the development of chlorosis. This decline was accompanied by changes in the leaf proteome, with 102 protein spots found to have altered intensities by 2-dimensional gel electrophoresis 12-36 h after toxin treatment. Forty-seven of these spots were identified by tandem mass spectrometry and included proteins involved in the light reactions of photosynthesis, the Calvin cycle, and the stress/defense response. These results suggest that Ptr ToxB disrupts photosynthesis in sensitive wheat, leading to oxidative stress and chlorophyll photooxidation. In another study, the *ToxB* gene encoding Ptr ToxB was transformed into a Ptr ToxB-non-producing isolate of *P. tritici-repentis*. The transformants induced chlorosis in a host-specific manner and caused significantly increased disease, with symptom severity correlated with the amount of Ptr ToxB produced. These findings indicate that the acquisition of toxin-producing ability is a sufficient condition for pathogenicity in *P. tritici-repentis*, and that Ptr ToxB confers virulence in a dosage-dependent manner. In a final study to explore the possible role(s) of *ToxB* homologs found in other ascomycetes, *Pyrenophora teres*, which causes net blotch of barley, was transformed with *ToxB*. Production of Ptr ToxB by *P. teres* increased virulence on

net blotch-resistant and susceptible barley cultivars. However, while the transformants exhibited an altered infection phenotype on toxin-sensitive wheat, they did not cause the typical chlorosis associated with tan spot. This suggests that while *ToxB* homologs may play a role in the pathogenicity of this fungus, production of Ptr ToxB is on its own not sufficient for *P. teres* virulence on wheat. Collectively, these studies improved understanding of the role of Ptr ToxB in tan spot development and fungal virulence. An enhanced knowledge of tan spot and related pathosystems will facilitate rational strategies for disease management.

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2-DE, two-dimensional gel electrophoresis

aa, amino acid

AdoMet, S-adenosyl-L-methionine

APX, ascorbate peroxidase

ATP, adenosine-5'-triphosphate

bp, base pairs

BSA, bovine serum albumin

DIG, digoxigenin

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

DTT, dithiothreitol

EDTA, ethylene diamine tetraacetic acid

ER, endoplasmic reticulum

ESI, electrospray ionization

ET, ethylene

ETS, effector-triggered susceptibility

FBA, fructose-bisphosphate aldolase

FNR, ferredoxin-NADP(H) oxidoreductase

FunCat, MIPS functional categories

GADPH, glyceraldehyde-3-phosphate dehydrogenase

GAP, glyceraldehyde-3-phosphate

GFP, green fluorescent protein

GLP, germin-like protein

GS, glutamine synthetase

His, hexahistidine

hph, *hygromycin B phosphotransferase*

HPLC, high performance liquid chromatography

HSP, heat shock protein

HST, host-specific toxin

IEF, isoelectric focusing

IPG, immobilized pH gradient

LB, Luria-Bertani

MALDI, matrix-assisted laser desorption/ionization

MDH, malate dehydrogenase

MIPS, Munich Information Center for Protein Sequences

M_r, relative molecular weights

MS/MS, tandem mass spectrometry

MWM, molecular weight marker

NCBInr, National Center for Biotechnology Information non-redundant

OEC, oxygen evolving complex

OEE1, oxygen-evolving enhancer protein 1

ORF, open reading frame

OXO, oxalate oxidase

PDA, potato dextrose agar

PDB, potato-dextrose broth

PEG, polyethylene glycol

PETC, photosynthetic electron transport chain

PGK, phosphoglycerate kinase

pI, isoelectric points

POD, peroxidise

PQ, plastoquinone

PRKase, phosphoribulokinase

PSI, photosystem I

PSII, photosystem II

PTH, phenylthiohydantoin

PVDF, polyvinylidene difluoride or polyvinylidene fluoride

Q_B, quinone B

q-TOF MS/MS, quadrupole-time-of-flight tandem mass spectrometry

RB, rehydration/sample buffer

RGD, arginine-glycine-aspartic acid; Arg-Gly-Asp

RGP, reversibly glycosylated polypeptide

RM, regeneration medium

RNA, ribonucleic acid

ROS, reactive oxygen species

RT, room temperature

RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase

SAS, Statistical Analysis System

sd-H_2O, sterile distilled water

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SNB, Stagonospora nodorum blotch

SOD, superoxide dismutase

SSC, saline-sodium citrate

STC, sorbitol/Tris/calcium

TBP, tributylphosphine

TBS, Tris-buffered saline

TCA, trichloroacetic acid

TIM, triosephosphate-isomerase

ToxABP1, ToxA Binding Protein 1

TTBS, Tween 20 (polyoxyethylene sorbitol monolaurate) in Tris-buffered saline

UV, ultraviolet

βME, 2-mercaptoethanol

1. Introduction

Tan spot is an economically important foliar disease of wheat caused by the necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Drechsler [anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker]. The pathogen produces at least three host-specific toxins, including the chlorosis-inducing Ptr ToxB, which plays an important role in tan spot development. In this thesis, I present the work that I have conducted over the past five years to improve understanding of the role of Ptr ToxB as a necrotrophic effector in the tan spot pathosystem. The thesis has been prepared in a manuscript format, and includes three research chapters (each representing a study in which I was the main author) and one appendix (representing a study to which I contributed substantially). The first study involves an assessment of the effect of Ptr ToxB on photosynthesis in a toxin-sensitive wheat genotype, and an analysis of Ptr ToxB-induced proteome changes in the leaves of this host at various times prior to the development of chlorosis symptoms. The second study involves the transfer of the *ToxB* gene, which encodes Ptr ToxB, into a Ptr ToxB-non-producing isolate of the fungus, in order to investigate the dual role of Ptr ToxB as a pathogenicity and virulence factor for *P. tritici-repentis*. The third study involves the transformation of the net blotch of barley pathogen, *Pyrenophora teres* Drechsler, with the *ToxB* gene in order to explore the possible role(s) of *ToxB* homologs found in various other ascomycete fungi. The appendix describes a comparison of the secretome and mycelial proteome of pathogenic and non-pathogenic isolates of *P. tritici-repentis*, in which I was responsible for the Western blotting and all related analyses.

1.1. Tan spot of wheat

The ascomycete fungus *P. tritici-repentis* was first described by Fries (1823) and later isolated from *Triticum repens* L. (couch grass) [syn. *Agropyron repens* (L.) P. Beauv.] by Diedicke (1902). In the United States, the fungus was first identified on quack grass near Madison, Wisconsin in the early 1920s (Drechsler, 1923). The occurrence of the fungus in Japan was first reported by Nishikado in 1928, when it was collected from *Agropyron semicostatum* (Nees ex Steud.) Boiss. (drooping wildrye) in the vicinity of Kurashiki, Okayama Prefecture. In Canada, *P. tritici-repentis* was first observed on 'Marquis' wheat in 1927 in Assiniboia, Saskatchewan (Conners, 1939) and on *Elymus canadensis* L. (Canada wild rye) in 1934 near Carman, Manitoba (Conners, 1934). In the United States, tan spot disease was first identified on wheat in New York in 1940 (Barrus, 1942). In Australia, it was recorded as 'yellow leaf spot disease of wheat' in 1950 and first reported in 1953 (Valder and Shaw, 1953).

Tan spot symptoms appear as tan-colored, oval-shaped spots or lesions containing a dark brown or black center, where sporulation of the fungus occurs. The lesions eventually coalesce and often become surrounded by chlorotic or yellow borders that may spread to cover the entire leaf. In resistant cultivars, symptoms are limited to the development of small dark flecks or spots, where sporulation may not occur (Hosford, 1982). Yield losses due to tan spot can reach up to 49% under conditions favorable for disease development (Rees *et al.*, 1982; Rees and Platz, 1983). According to Shabeer and Bockus (1988), the factors affecting yield losses include the level of inoculum, duration of the wet period

after inoculation, host genotype and the growth stage of the wheat. The disease was not severe in Canada and Australia until the 1970s (Rees and Platz, 1979; Tekauz, 1976). The emergence of tan spot as a serious disease at that time may have resulted largely from changes in cultivation practices by farmers, who shifted from conventional to reduced tillage systems that leave crop residues on the soil surface (Bockus and Shroyer, 1998; Rees and Platz, 1992). Historically, farmers had burnt wheat residues or extensively worked the soil, burying infected plant debris beneath the surface. Since *P. tritici-repentis* is a stubble-borne pathogen, the retention of crop residues allowed survival of large amounts of inoculum. Recently, Oliver *et al.* (2008) reported that the majority of the cultivars planted in Australia were susceptible to tan spot before and during the period of disease emergence and spread, providing additional support for the suggestion that it was changes in stubble management practices that were most important in the emergence of tan spot as a major disease of wheat.

1.2. *Pyrenophora tritici-repentis*

1.2.1. Taxonomy, synonyms and nomenclature

The tan spot fungus belongs to Kingdom: *Fungi*; Phylum: *Ascomycota*; Subphylum: *Pezizomycotina*; Class: *Dothideomycetes*; Order: *Pleosporales*; Family: *Pleosporaceae*; Genus: *Pyrenophora*; Species: *tritici-repentis* (Erikson and Winka, 1997; Fries, 1849; Schoch *et al.*, 2009). However, the fungus has been historically referred to by various names. Names for the teleomorph have included: *Sphaeria trichostoma* Fr., *Pleospora trichostoma* (Fr.) Ces. & De Not., *Pyrenophora trichostoma* (Fr.) Fckl., *Pleospora tritici-repentis* Died., *Pleospora*

trichostoma f. sp. *tritici-repentis* (Died.) Noack, *Pyrenophora tritici-repentis* (Died.) Drechs., and *Pyrenophora tritici-vulgaris* Dickson (De Wolf *et al*., 1998; Hosford, 1982). The anamorph has been known as: *Helminthosporium gramineum* Rab. ex. Schlecht f. sp. *tritici-repentis* Died., *H. tritici-repentis* (Died.) Died., *H. tritici-vulgaris* Nisikado, *Drechslera tritici-vulgaris* (Nisikado) Ito., and *D. tritici-repentis* (Died.) Shoem. (De Wolf *et al*., 1998; Hosford, 1982).

The genus *Pyrenophora* Fr. was established by Fries (1849) and the word *Pyrenophora* means 'bearing a kernel, seed or pit' within the ascocarp (Shoemaker, 1961). The fungus was recovered from *Triticum repens* L. and reported as a specialized form, *Helminthosporium gramineum* f. sp. *tritici-repentis*, by Diedicke (1902). Drechsler (1923) also collected the fungus from *Triticum repens* L. and assigned it the teleomorph name *Pyrenophora tritici-repentis*. Since then, the hyphenated species name (*tritici-repentis*) has continued to be used. Shoemaker (1959) separated the anamorphic genus *Helminthosporium* into three genera (*Drechslera*, *Bipolaris* and *Helminthosporium*) based on differences in conidial germination, placing the tan spot fungus within the genus *Drechslera*. Later, Shoemaker (1961) concluded that the appropriate classification of the perfect state of the fungus was in the genus *Pyrenophora*, not in the genus *Pleospora*, after he identified ascospores and conidiophores with single conidia on the specimen from the Fries Herbarium. Shoemaker also considered all the synonyms of the imperfect state of the fungus as *Drechslera tritici-repentis* (Shoemaker, 1962). Currently, the unanimously accepted names are *P. tritici-*

repentis for the teleomorph and *D. tritici-repentis* for the anamorph (De Wolf *et al*., 1998).

1.2.2. Host range

P. tritici-repentis can attack most durum (*Triticum turgidum* L. var. *durum*) and common wheat (*Triticum aestivum* L.) genotypes, which are considered as the principal hosts of the pathogen because of their agricultural and economic importance. However, the fungus has a very wide host range and has been isolated from various grass species: *Agropyron desertorum* (Fisch. ex Link) Schult. (desert wheatgrass), *A. intermedium* (Host) Barkworth & D.R. Dewey (intermediate wheatgrass), *A. cristatum* (L.) Gaertn. (crested wheatgrass), *A. repens* (syn. *Elymus repens* (L.) Gould; quack grass), *A. smithii* Rydb. (western wheatgrass), *Alopecurus pratensis* L. (meadow foxtail), *Bromus inermis* Leyss. (smooth bromegrass), *Critesion jubatum* (L.) Nevski (wild barley), *Danthonia intermedia* Vasey (timber oatgrass), *Echinochloa crusgalli* (L.) P. Beauv. (barnyard grass), *Elymus angustus* (Trin.) Pilg. (altai wild-rye), *E. canadensis* (Canada wild-rye), *Leymus racemosus* (Lam.) Tzvelev (mammoth wild-rye), *Psathyrostachys juncea* (Fisch.) Nevski (Russian wild-rye), *Secale cereale* L. (cereal rye), *Triticum araraticum* Jakubz. (wild wheat), *T. carthlicum* Nevski (Persian wheat), *T. dicoccoides* L. (wild emmer wheat) (Hosford, 1971; Krupinsky, 1982, 1992a; Ali and Francl, 2003). In addition, *P. tritici-repentis* may saprophytically colonize barley (*Hordeum vulgare* L.) and could be recovered from barley leaves (Krupinsky, 1992b; Summerell and Burgess, 1988). The pathogen population on these alternative hosts might differ in aggressiveness

and may be more or less pathogenic on its agriculturally important wheat host (De Wolf *et al.*, 1998; Krupinsky, 1992b). Because of its ability to survive on various grass species, the secondary hosts of *P. tritici-repentis* may serve as a source of primary inoculum between wheat growing seasons. It also is possible that the pathogen population on different grass species may serve as a source of fungal genetic variation (De Wolf *et al.*, 1998). Indeed, Strelkov and Lamari (2003) hypothesized that *P. tritici-repentis* may have evolved on grass species before moving to its wheat host.

1.2.3. Race classification

Pyrenophora tritici-repentis can differentially induce combinations of two distinctive symptoms on susceptible wheat genotypes: tan necrosis and extensive chlorosis (Lamari and Bernier, 1989a). Lamari and Bernier (1989b) initially grouped isolates of the pathogen into three pathotypes based on the differential reactions of 11 wheat cultivars. Later, a fourth pathotype was identified with the addition of two new wheat genotypes to the differential set (Lamari *et al.*, 1991). Pathotype 1 consisted of isolates that induced both necrosis and chlorosis; pathotypes 2 and 3 consisted of isolates that caused only necrosis or chlorosis, respectively; and pathotype 4 consisted of isolates that could not produce either symptom. However, the limitations of the pathotype classification system were highlighted when fungal isolates were identified from eastern Algeria that could induce the chlorosis symptom, but on different host genotypes than other pathotype 3 isolates (Lamari *et al.*, 1995). While the pathotype 3 isolates characterized earlier from Canada caused chlorosis on the wheat line 6B365 but

not on line 6B662, those from Algeria caused chlorosis on line 6B662 but not on line 6B365. Therefore, Lamari *et al.* (1995) proposed a race classification system, in which isolates of *P. tritici-repentis* were grouped into races based on their virulence on a host differential set, as opposed to their ability to generically cause necrosis or chlorosis.

To date, at least eight races of *P. tritici-repentis* have been identified based on the reactions of three effective wheat differentials, consisting of 'Glenlea', line 6B365 and line 6B662 (Lamari *et al.*, 1995, 2003; Strelkov *et al.*, 2002). For practical reasons, isolates of pathotypes 1 to 4 were renamed races 1 to 4, respectively, while the new North African isolates were designated as race 5 (Lamari *et al.*, 1995). The existence of race 6 was reported after analyzing additional isolates collected in eastern Algeria (Strelkov *et al.*, 2002); race 6 isolates are able to produce chlorosis on lines 6B365 and 6B662, and therefore combine the virulence patterns of races 3 and 5. Later, two additional races were identified from the host center of diversity and designated as races 7 and 8 (Lamari *et al.*, 2003). Race 7 isolates combine the virulence patterns of races 2 and 5, while race 8 isolates combine the virulence patterns of races 2, 3 and 5 and therefore have the widest host range. The eight known races of *P. tritici-repentis* and the reaction of the three effective wheat differentials are summarized in Table 1-1.

Table 1-1. Reactions of three effective wheat differential hosts to the eight characterized races of *Pyrenophora tritici-repentis* (adapted from Lamari and Strelkov, 2010).

Host Genotype	Race 1	Race 2	Race 3	Race 4	Race 5	Race 6	Race 7	Race 8
'Glenlea'	S (N)	S (N)	R	R	R	R	S (N)	S (N)
6B662	R	R	R	R	S (C)	S (C)	S (C)	S (C)
6B365	S (C)	R	S (C)	R	R	S (C)	R	S (C)

Note: S (N) = susceptible (necrotic reaction); S (C) = susceptible (chlorotic reaction); R = resistant.

While races 1, 2, 3, 4 and 5 have all been reported from North America, more than 90% of the *P. tritici-repentis* isolates from this continent are classified as races 1 or 2 (Lamari and Strelkov, 2010). Races 5 and 6 have been reported from North Africa, while races 1, 2, 3, 5, 7 and 8 were found in a survey of the Caucasus and the Fertile Crescent regions (Lamari *et al.*, 2005; Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). Limited surveys of central Asia revealed the presence of races 1 and 2 in that region (Lamari *et al.*, 2005). It is likely that the current differential set does not completely capture the extent of pathogenic variation in *P. tritici-repentis*, particularly in areas where populations might be particularly diverse, such as the host centre of diversity. Therefore, the differential set may eventually have to be expanded or modified, perhaps by adding local wheat varieties or land races from the Caucasus and the Fertile Crescent (Lamari *et al.*, 2005).

1.3. Effectors

A broad and inclusive definition of effectors is all pathogen proteins and small molecules that alter or manipulate host-cell structure and function (Hogenhout *et al.*, 2009). Effectors can be involved in either facilitating infection (virulence factors, degradative enzymes and toxins) or triggering host defense responses (avirulence factors and elicitors) or both (Hogenhout *et al.*, 2009). The term effector is a neutral word and does not imply a positive or a negative impact on the interaction between plant host and parasite, so it has gained in popularity in recent years (Hogenhout *et al.*, 2009; Kamoun, 2006). To date, it has been observed that known effector proteins from filamentous fungal pathogens have at

least one of the following eight features: 1) contain a secretion signal peptide, 2) are encoded by *in planta* induced genes, 3) posses similarity to haustorial proteins, 4) are small cysteine-rich proteins, 5) have a known effector motif or nuclear localization signals, 6) are internal repeat-containing proteins, 7) are encoded by genes with long flanking intergenic regions, and/or 8) do not contain Pfam domains, except for proteins associated with pathogenicity (Saunders *et al.*, 2012). Therefore, effectors are encoded by genes that reside in pathogen genomes, but the gene products actually function at the interface with the host plant or even inside the host plant tissues (Kamoun, 2007).

Host-specific toxins (HSTs) are a special subclass of effectors produced by necrotrophic fungal pathogens (Friesen *et al.*, 2008a). They are defined as metabolic products or molecules produced by pathogenic microorganisms that are selectively toxic only to certain susceptible host genotypes. These HSTs have little effect on non-hosts, and non-host plants are tolerant to the HSTs (Pringle and Scheffer, 1964; Scheffer and Livingston, 1984). Classically, pathogenicity is a comprehensive and qualitative term to refer the ability of a microorganism to cause disease, while virulence is a quantitative term to describe the amount or extent of disease or damage caused to a specific host genotype by a given pathogen (Shaner *et al.*, 1992; Yoder, 1980). Therefore, with regards to HSTs, investigators shall need a clear practical distinction between what is disease and what is not disease, which varies between different pathosystems, in order to evaluate pathogenicity factors. In addition, precise and reproducible methods for quantifying disease are required to evaluate virulence factors (Yoder, 1980). As

HSTs produced by pathogens facilitate disease and the hosts produce receptors required for susceptibility, HSTs have been known to mediate and establish compatible interactions between the host and the pathogen, resulting in disease development (Walton, 1996). Some HSTs that are known to serve as necrotrophic effectors include: victorin (a cyclic pentapeptide) produced by *Cochliobolus victoriae* R.R. Nelson, T-toxin (a polyketide) produced by race T isolates of *C. heterostrophus* (Drechs.) Drechs., HC-toxin (a cyclic tetrapeptide) produced by race 1 isolates of *C. carbonum* R.R. Nelson, and AAL-toxin (an aminopentol ester) produced by *Alternaria alternata* f.sp. *lycopersici* (Markham and Hille, 2001; Oliver and Solomon, 2010; Wolpert *et al.*, 2002). The first proteinaceous HSTs were identified from *P. tritici-repentis*, with the eight races of the fungus known to produce at least three toxins (Ptr ToxA, Ptr ToxB and Ptr ToxC). These HSTs, which will be described in more detail in the following section, are largely responsible for the development of the tan necrosis and chlorosis symptoms associated with tan spot (Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). Other necrotrophic effectors, associated with the Stagonospora nodorum blotch of wheat pathosystem, will also be discussed.

1.3.1. Necrotrophic effectors of *P. tritici-repentis*

1.3.1.1. Ptr ToxA

Ptr ToxA was the first HST identified from *P. tritici-repentis*. It causes necrosis on sensitive wheat genotypes and has been the most extensively studied of the Ptr toxins (Ciuffetti *et al.*, 1998; Ballance *et al.*, 1989; Tomás *et al.*, 1990; Tuori *et al.*, 1995; Zhang *et al.*, 1997). Although originally identified from races 1

and 2 of *P. tritici-repentis*, it has since been found to be produced by races 7 and 8 as well (Lamari *et al.*, 2003). Sensitivity to Ptr ToxA in host genotypes is conferred by a single locus gene (*Tsn1*) found on the long arm of wheat chromosome 5B (Faris *et al.*, 1996; Gamba *et al.*, 1998). The role of Ptr ToxA as a pathogenicity factor for *P. tritici-repentis* was first suggested by Lamari and Bernier (1989a). An independent study by Ciuffetti *et al.* (1997) provided support for this hypothesis, when it revealed that transformation of a non-pathogenic isolate with the gene encoding Ptr ToxA enabled the transformed isolate to cause disease on a Ptr ToxA-sensitive host. In contrast, Friesen *et al.* (2002) suggested that Ptr ToxA could be viewed as a virulence factor, after they found that some race 1 isolates of *P. tritici-repentis* induced lesions on toxin-insensitive mutant wheat lines. Friesen *et al.* (2003) also suggested that Ptr ToxA may serve as a virulence factor, rather than as a pathogenicity factor, for race 2 of the fungus, since they found that not all host genotypes are sensitive to Ptr ToxA. Regardless of whether Ptr ToxA is a pathogenicity and/or virulence factor for *P. tritici-repentis*, it is clear that this toxin is a HST and a determinant of host range, since its production is a sufficient condition for pathogenicity on some host genotypes carrying the toxin-sensitivity gene (Ciuffetti *et al.*, 1997; Strelkov and Lamari, 2003).

Ptr ToxA is a small protein encoded by a single-copy gene termed *ToxA*. This gene encodes a pre-pro-protein comprised of 178 amino acids with a predicted mass of 19.7 kDa (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). At the N-terminus, the pre-pro-protein contains a 22 amino acid (aa) signal peptide

sequence (pre-region) for protein secretion and a 4.3 kDa pro-region (aa residues 23-60) required for proper folding (Tuori *et al.*, 2000). These are cleaved to produce an active and mature Ptr ToxA protein (residues 61-178, C-terminus) 13.2 kDa in mass (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Two cysteine residues (Cys64 and Cys160) in the C-terminus of the mature Ptr ToxA form a disulfide bond that is essential for fully functional toxin activity (Sarma *et al.*, 2005; Tuori *et al.*, 2000). The Ptr ToxA protein also contains multiple motifs necessary for Ptr ToxA activity, including two putative casein kinase 2 phosphorylation sites and a vitronectin-like motif with an Arg-Gly-Asp (RGD) sequence for cell adhesion (Manning *et al.*, 2004; Meinhardt *et al.*, 2002). Resolution of the crystal structure of Ptr ToxA revealed that this RGD domain is located on a mobile loop that may be involved in protein-protein interactions with a host receptor (Sarma *et al.*, 2005).

Microscopy and localization studies with green fluorescent protein (GFP)-tagged Ptr ToxA revealed that the toxin is internalized only in toxin-sensitive wheat cultivars, without disrupting the integrity of the plasma membrane (Manning and Ciuffetti, 2005). While the exact mechanism of internalization of Ptr ToxA is not clear, Manning and Ciuffetti (2005) suggested that it may enter the host cells via receptor mediated endocytosis. Once internalized, Ptr ToxA was found to localize to cytoplasmic compartments and chloroplast mesophyll cells. The exact mechanism of action of the toxin is not known, although Ptr ToxA-induced necrosis requires light (Manning and Ciuffetti, 2005). Intracellular expression of the toxin by biolistic bombardment of the cells of Ptr ToxA-

sensitive and insensitive genotypes resulted in the death of both cell types, suggesting that the internal site of Ptr ToxA action was present in both (Manning and Ciuffetti, 2005). Manning *et al.* (2007) employed a yeast two-hybrid assay and found that Ptr ToxA, once internalized, interacts with a chloroplast membrane protein, designated ToxA Binding Protein 1 (ToxABP1). ToxABP1 is present in the membranes and stroma of the chloroplasts, although Ptr ToxA appears to interact primarily with ToxABP1 associated with the membrane (Manning *et al.*, 2007). Although the exact role of ToxABP1 is not known, it has been suggested that it might have a function in the assembly and disassembly of photosystem II (PSII) complexes or thylakoid formation (Keren *et al.*, 2005; Wang *et al.*, 2004). Therefore, the interaction of Ptr ToxA with ToxABP1 would lead to the disruption of PSII and/or thylakoid structures resulting in oxidative stress (Keren *et al.*, 2005; Manning *et al.*, 2009; Wang *et al.*, 2004).

Another research group also applied a yeast two-hybrid approach to identify the host target protein for Ptr ToxA. Tai *et al.* (2007) reported that wheat plastocyanin, designated TaPCN, is the target protein of Ptr ToxA. Plastocyanin is a nuclear encoded chloroplast protein in the electron transport chain of photosynthesis, and transfers electrons between photosystem I (PSI) and PSII. This group further supported their findings by virus-induced gene silencing of wheat plastocyanin, which resulted in cell death in a manner similar to Ptr ToxA-induced necrosis. A possible reason for the discrepancy between the two groups in the proposed targets is that Manning *et al.* (2009) used a construct for Ptr ToxA expression that included the pro-region (N-domain) required for proper folding of

the mature toxin (Tuori *et al.*, 2000), while Tai *et al.* (2007) used only the mature toxin (C-domain).

Most recently, Faris *et al.* (2010) demonstrated that transcription of *Tsn1* is tightly regulated by the circadian clock and light, supporting previous findings of the light dependency of Ptr ToxA-induced necrosis and its association with photosynthetic pathways (Manning and Ciuffetti, 2005). However, since yeast two-hybrid experiments revealed that the Tsn1 protein does not interact directly with Ptr ToxA, Tsn1 is not likely to be the receptor of Ptr ToxA (Faris *et al.*, 2010). Faris *et al.* (2010) also found in their preliminary yeast two-hybrid analysis that Tsn1 does not interact directly with either ToxABP1 or plastocyanin. The Tsn1 protein does not contain any transmembrane domains and appears to be located within the cell. Therefore, Faris *et al.* (2010) hypothesized that Tsn1 mediates Ptr ToxA recognition indirectly, by interacting with Ptr ToxA and initiating the signaling required for importation of the toxin via the endocytic process. This eventually results in the accumulation of Ptr ToxA and necrosis in the toxin-sensitive host tissues.

Manning *et al.* (2008) proposed a model for Ptr ToxA action, in which the toxin binds to a high-affinity receptor via the RGD-containing loop and Ptr ToxA is internalized through receptor-mediated endocytosis into an endosome. Once internalized, the receptor is dissociated from Ptr ToxA and recycled back to the membrane, while Ptr ToxA escapes the endosome and enters the cytosol. Ultimately, the toxin binds to the chloroplast, the final site of action, resulting in irreversible alteration in function and cell death. Light-induced cell death after

treatment with Ptr ToxA is explained by a decrease in PSI and PSII proteins, affecting photosynthetic electron transport, as well as by a decline in the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), leading to the accumulation of reactive oxygen species (ROS) in the chloroplasts (Manning *et al.*, 2009).

1.3.1.2. Ptr ToxB

Ptr ToxB is a chlorosis-inducing, necrotrophic effector first purified from a race 5 isolate of *P. tritici-repentis*, but now also known to be produced by races 6, 7 and 8 of the fungus (Strelkov *et al.*, 1999; Strelkov and Lamari, 2003; Strelkov *et al.*, 2002, 2006). Sensitivity to Ptr ToxB in the host is conferred by the *Tsc2* gene, which is located on the short chromosome arm 2BS in tetraploid (*Triticum turgidum* L.) and hexaploid (*Triticum aestivum* L.) wheat (Abeysekara *et al.* 2010; Friesen and Faris, 2004). The toxin is a small, hydrophilic, heat-stable protein (Orolaza *et al.*, 1995; Strelkov *et al.*, 1999). The molecular mass of Ptr ToxB was originally determined to be 6,612 ± 7 Da by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Strelkov *et al.*, 1999), but has been more recently measured as 6,538 ± 2 Da using the same technique (Figueroa Betts *et al.*, 2011). This small difference may reflect improvements in MALDI-TOF MS techniques over the past decade (Figueroa Betts *et al.*, 2011).

Strelkov *et al.* (1999) were the first to purify Ptr ToxB and obtained a partial N-terminus amino acid sequence of the protein, which enabled the encoding gene (*ToxB*) to be cloned by several independent research groups

(Martinez *et al.*, 2001; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006). The *ToxB* gene consists of a 261 bp open reading frame (ORF) with no introns, and encodes an 87 aa pre-protein that includes a 23 aa signal peptide. Following proteolytic cleavage, Ptr ToxB is secreted by *P. tritici-repentis* as a mature protein 64 aa in length (Martinez *et al.*, 2001; Strelkov and Lamari, 2003). In contrast to *ToxA*, *ToxB* has been found to exist as a multiple copy gene in isolates of *P. tritici-repentis* that possess Ptr ToxB activity (Lamari *et al.*, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). For example, Southern blotting analysis revealed 8-10 copies of the gene in a highly pathogenic race 5 isolate (Alg3-24) from Algeria (Strelkov *et al.*, 2006) and nine copies in another highly pathogenic race 5 isolate (DW7) from the United States (Martinez *et al.*, 2004). A weakly pathogenic race 5 isolate from Canada (92-171R5) was found to possess two copies of the gene (Strelkov *et al.*, 2006). Interestingly, homologs of *ToxB* are also found as single copies in isolates belonging to races 3 and 4 of *P. tritici-repentis* (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006). Although these races do not appear to possess any Ptr ToxB activity, the *ToxB* homologs are active genes and are transcribed at low levels (Amaike *et al.*, 2008; Strelkov *et al.*, 2006).

The *ToxB* homolog present in race 4 isolates of *P. tritici-repentis*, termed *toxb* by Martinez *et al.* (2004), shows 86% homology to the 'wild-type' (race 5) *ToxB* over the ORF (Martinez *et al.*, 2004; Strelkov and Lamari, 2003). The form of the toxin encoded by *toxb* has a 23 aa signal peptide and four cysteine residues, like the wild-type, but contains one more aa residue in the mature protein region with 13 aa substitutions (Figueroa Betts *et al.*, 2011; Martinez *et al.*, 2004;

Strelkov and Lamari, 2003). Despite the high similarity between the encoded proteins, Ptr ToxB from race 4 induces only trace or no chlorosis on Ptr ToxB-sensitive wheat leaves, suggesting that the few differences in sequence relative to the wild-type are sufficient to cause a loss of toxin activity (Kim and Strelkov, 2007; Figueroa Betts *et al.*, 2011). In addition to the *ToxB* homologs in races 3 and 4 of *P. tritici-repentis*, other *ToxB* homologs have recently been identified in *Pyrenophora bromi* (Died.) Drechsler and other ascomycetes, including *P. teres*, *P. graminea* S. Ito & Kurib., *P. lolii* Dovaston, *Alternaria alternata* (Fr) Keissl., *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur, *C. victoriae* R.R. Nelson and *Magnaporthe grisea* (T.T. Hebert) M.E. Barr (Andrie *et al.*, 2008). Therefore, it appears that *ToxB* and its homologs may have originated in an early ancestor of the Ascomycota (Andrie *et al.*, 2008). This suggestion is consistent with the hypothesis that *P. tritici-repentis* and its toxins evolved on grass species before moving to its current wheat host (Strelkov and Lamari, 2003). However, the role(s) of *ToxB* homologs in non-pathogens of wheat and in races of *P. tritici-repentis* that lack Ptr ToxB activity is unknown and awaits further study.

With respect to the ability of wild-type Ptr ToxB to induce chlorosis on toxin-sensitive wheat genotypes, the N-terminus region of the protein was found to play a particularly important role, although the entire protein is required to induce full chlorosis symptom development (Figueroa Betts *et al.*, 2011). While the three-dimensional structure of Ptr ToxB has yet to be determined, Figueroa Betts *et al.* (2011) reported that one of the amino acids surrounding the first cysteine residue, valine, is required for proper folding of the protein and plays a

critical role in the formation of a disulfide bond, which may be important for toxin activity. However, the physiological and biochemical mechanisms for this activity in toxin-sensitive wheat tissue are not completely understood.

It appears that the chlorosis induced by Ptr ToxB results from chlorophyll degradation, rather than an inhibition of chlorophyll synthesis, since toxin-treatment does not interfere with chlorophyll accumulation in etiolated seedlings (Strelkov *et al.*, 1998). As with Ptr ToxA, Ptr ToxB activity is also light-dependent, suggesting that chlorosis results from the photochemical bleaching of chlorophyll molecules by ROS (Strelkov *et al.*, 1998). This suggestion was supported by the incubation of Ptr ToxB-treated leaves with ROS scavengers, one of which (p-benzoquinone) prevented the development of chlorosis (Strelkov *et al.*, 1998). High performance liquid chromatography (HPLC) chlorophyll degradation profiles were also consistent with photo-oxidation of the chlorophyll into low molecular mass compounds (Strelkov *et al.*, 1998). The carotenoid concentration in affected tissues also declined, but this decline was concurrent with and of a smaller magnitude than the decline in chlorophyll, indicating that chlorosis did not result from a deficiency in these photo-protective pigments. It was suggested that the increase in ROS and the subsequent photo-oxidation of the chlorophyll in Ptr ToxB-treated tissues results from an inhibition of photosynthesis, as illuminated thylakoid membranes became unable to dissipate the excitation energy normally used to drive photosynthetic electron transport (Strelkov *et al.*, 1998). However, no direct evidence was provided to support this hypothesis. The lack of information on the mechanism of Ptr ToxB action

prompted us to undertake the studies presented in Chapter 2 of this thesis, in which the effect of the toxin on photosynthesis and the foliar proteome of a toxin-sensitive wheat genotype is examined.

1.3.1.3. Ptr ToxC

The third HST produced by *P. tritici-repentis* is Ptr ToxC, which is the least characterized Ptr effector and also induces extensive chlorosis, but on different host genotypes than Ptr ToxB (Strelkov and Lamari, 2003). Ptr ToxC is believed to be synthesized by races 1, 3, 6 and 8 of *P. tritici-repentis*, although it has not been purified to homogeneity. Therefore, much of the evidence for its existence comes from genetic studies and the distinct chlorosis symptoms induced on sensitive hosts. Sensitivity to Ptr ToxC in wheat is conferred by the *Tsc1* gene, located on the short arm of chromosome 1A (Effertz *et al.*, 2002). The toxin was partially characterized by gel filtration, ion-exchange and reversed-phase chromatography, and reported to be a low molecular mass molecule with non-ionic and polar properties (Effertz *et al.*, 2002). While Ptr ToxC appears to be stable at various pH values (pH 2 to pH 10), it is not heat-stable (Effertz *et al.*, 2002). There are no reports in which the mode of action of Ptr ToxC was studied, perhaps because of the inherent difficulties in working with a non-purified toxic principle.

In addition to Ptr ToxA, Ptr ToxB and Ptr ToxC, there have been preliminary reports of the existence of other HSTs produced by *P. tritici-repentis* (Manning *et al.*, 2002; Meinhardt *et al.*, 2003). However, evidence for these

putative HSTs has not been provided in the refereed literature and they await further characterization.

1.3.1.4. Ptr toxin production and the race classification of *P. tritici-repentis*

The races of *P. tritici-repentis* are defined by their ability to produce the various Ptr toxins, which in turn results in chlorosis and/or necrosis symptom development on the three effective wheat differential hosts, 'Glenlea', 6B365 and 6B662 (Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). As such, races of the fungus have simple or complex virulence patterns, based on the number of toxins that they produce. Race 1 isolates of *P. tritici-repentis*, which produce both Ptr ToxA and Ptr ToxC, can cause disease on the wheat differentials 'Glenlea' (sensitive to ToxA) and 6B365 (sensitive to ToxC). Race 2 isolates, which produce only Ptr ToxA, can only cause disease on 'Glenlea', while race 3 isolates, which produce only Ptr ToxC, can only cause disease on 6B365. Race 4 isolates do not produce any known toxins and are considered non-pathogenic in the tan spot pathosystem. Race 5 isolates produce only Ptr ToxB and can cause disease only on 6B662 (sensitive to ToxB). Race 6 isolates, which produce both Ptr ToxB and Ptr ToxC, can cause disease on 6B662 and 6B365. Isolates of race 7, which produce Ptr ToxA and Ptr ToxB, can cause disease on 'Glenlea' and 6B662. Finally, race 8 isolates of *P. tritici-repentis* produce all three Ptr toxins and can therefore attack all three wheat differentials (Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). Toxin production by the eight known races of *P. tritici-repentis* is summarized in Table 1-2. It is likely that as the existence of other Ptr

Table 1-2. Production of host-specific toxins by the eight characterized races of *Pyrenophora tritici-repentis* (reproduced from Lamari and Strelkov, 2010).

Race	Toxins	Toxin genes or homologues
1	Ptr ToxA, Ptr ToxC	*ToxA*, (*ToxC*?)[1]
2	Ptr ToxA	*ToxA*
3	Ptr ToxC	*ToxB* homolog[2], (*ToxC*?)
4	none	*ToxB* homolog[3] (= *toxb*)
5	Ptr ToxB	*ToxB*
6	Ptr ToxB, Ptr ToxC	*ToxB*, (*ToxC*?)
7	Ptr ToxA, Ptr ToxB	*ToxA*, *ToxB*,
8	Ptr ToxA, Ptr ToxB, Ptr ToxC	*ToxA*, *ToxB*, (*ToxC*?)

Notes: [1]The gene(s) involved in the synthesis of Ptr ToxC are unknown.

[2]Although race 3 possesses a *ToxB* homolog, it does not exhibit any Ptr ToxB activity (Strelkov *et al.*, 2006).

[3]Although race 4 possesses a *ToxB* homolog, it does not exhibit any Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov *et al.*, 2006); the race 4 homolog was termed *toxb* by Martinez *et al.* (2004).

toxins is conclusively demonstrated, and/or additional effective differentials are found, more races of the pathogen will be identified.

1.3.2. Other necrotrophic effectors

Stagonospora nodorum (Berk.) Castell. & Germano [teleomorph: *Phaeosphaeria nodorum* (Müll.) Hedjar.] is the causal agent of Stagonospora nodorum blotch (SNB), which is a major foliar and glume disease of common and durum wheat as well as other cereals and wild grasses. *S. nodorum* is a necrotrophic phytopathogen that causes the development of oval-shaped lesions, often surrounded by a chlorotic halo, on susceptible host genotypes. The fungus is closely related to *P. tritici-repentis*, belonging to the class Dothideomycetes and the order Pleosporales. Like *P. tritici-repentis*, *S. nodorum* is now known to produce multiple proteinaceous HSTs (Friesen and Faris, 2010; Solomon *et al.*, 2006). To date, five necrotrophic effectors have been reported from this pathogen. The first HST identified from *S. nodorum*, SnTox1, was reported by Liu *et al.* (2004a), who isolated it from culture filtrates of the fungus. SnTox1 is a small proteinaceous molecule. Sensitivity to SnTox1 in wheat is conferred by a single dominant gene, designated *Snn1*, located at the distal end of the short arm of chromosome 1B (1BS). Like the Ptr toxins in the tan spot pathosystem, SnTox1 plays an important role in SNB development and is believed to be a major virulence factor for *S. nodorum* (Liu *et al.*, 2004a, 2004b). Recently, Liu *et al.* (2012) cloned the *SnTox1* gene that encodes SnTox1 using a bioinformatics approach, which was based on the annotated *S. nodorum* genome and features common to effector molecules. They found that *SnTox1* encodes a 117 aa protein,

including a predicted 17 aa signal peptide. After cleavage of the signal peptide, the mature SnTox1 protein is an estimated 10.33 kDa in size. Interestingly, mature SnTox1 contains 16 cysteine residues; a high number of cysteine residues is a common trait of avirulence effectors produced by *Cladosporium fulvum* Cooke, *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hans and *Rhynchosporium secalis* (Oud.) J.J. Davis. Liu *et al.* (2012) also demonstrated that light is required for SnTox1-induced necrosis and for fungal penetration during the infection process, with SnTox1 playing an important role during penetration.

The second HST from *S. nodorum* was reported by Friesen *et al.* (2006). This toxin, designated SnToxA, and the gene encoding the protein, *SnToxA*, were found to share more than 99% homology with ToxA from *P. tritici-repentis*. SnToxA also appears to be a major factor in the development of SNB on wheat genotypes that carry the *Tsn1* gene. As noted earlier, *Tsn1* is located in the long arm of chromosome 5B (5BL) and confers sensitivity to Ptr ToxA (Faris *et al.*, 1996; Stock *et al.*, 1996). Liu *et al.* (2006) demonstrated that SnToxA is functionally identical to Ptr ToxA. Unlike the *ToxA* gene in *P. tritici-repentis* which is found as a single copy, *SnToxA* is a multi-copy gene, and Friesen *et al.* (2006) suggested that the tan spot fungus may have acquired *ToxA* from *S. nodorum* through horizontal gene transfer.

The third HST identified from *S. nodorum* has been termed SnTox2 and is also a protein, between 7 and 10 kDa in mass (Friesen *et al.*, 2007). SnTox2 induces extensive necrosis on susceptible wheat genotypes. Sensitivity to SnTox2

in wheat is conferred by a single dominant gene, designated *Snn2*, which is located to the short arm of wheat chromosome 2D (2DS) (Friesen *et al.*, 2007). The gene encoding SnTox2 has not yet been described in the literature.

The fourth HST reported from *S. nodorum* is designated as SnTox3, and is also a small necrosis-inducing protein (Friesen *et al.*, 2008b). The corresponding wheat sensitivity/susceptibility gene, *Snn3*, has been identified and is located near the telomere of the short arm of wheat chromosome 5B (5BS). The gene encoding SnTox3 was cloned by Liu *et al.* (2009) and designated as *SnTox3*. The *SnTox3* gene is 693 bp in length, contains no introns and has no known homologs. It encodes a 230 aa pre-protein with a calculated mass of 25.85 kDa. The first 20 aa residues constitute a predicted signal peptide for secretion, and the mature SnTox3 contains 6 cysteine residues (Liu *et al.*, 2009). Unlike the additive effects observed for the SnToxA-*Tsn1*/SnTox1-*Snn1* and the SnToxA-*Tsn1*/SnTox2-*Snn2* interactions, it appears that both the SnToxA-*Tsn1* and SnTox2-*Snn2* interactions are epistatic to the SnTox3-*Snn3* interaction, highlighting the complexity of wheat-SNB pathosystem (Chu *et al.*, 2010; Friesen *et al.*, 2008b).

Recently, a sensitivity gene to the SnTox3 effector was identified in the diploid *Aegilops tauschii* (wild goatgrass), a D-genome donor to common wheat, which was mapped to the short arm of chromosome 5D (5DS) (Zhang *et al.*, 2011). Therefore, the homeologous sensitivity genes were renamed *Snn3-B1* in wheat and *Snn3-D1* in *A. tauschii*. It appears that the SnTox3-*Snn3-D1* interaction in *A. tauschii* is more severe than the SnTox3-*Snn3-B1*interaction in wheat, suggesting different affinities for SnTox3 recognition and signal transduction

(Zhang *et al.*, 2011). Further characterization of how these homeologous genes mediate recognition of SnTox3 is needed to better understand effector-host interactions in the wheat-*S. nodorum* pathosystem.

The fifth HST reported from *S. nodorum* has been termed SnTox4. Like the other effectors reported from this pathogen, SnTox4 is a protein, with an estimated size of 10-30 kDa. The toxin induces a distinct mottled necrosis on sensitive host genotypes, and also requires light for symptom development (Abeysekara *et al.*, 2009). Sensitivity to SnTox4 is conferred by a single dominant gene, designated *Snn4*, which maps to the short arm of wheat chromosome 1A (1AS) (Abeysekara *et al.*, 2009). To date, the gene encoding SnTox4 has not been cloned, and no information is available on how this effector interacts with the host or causes the mottled necrosis symptom on affected leaves.

Given the large number of necrotrophic effectors or HSTs now identified from the tan spot and SNB pathosystems, it is likely that necrotrophic effectors play roles in other cereal diseases as well. This would seem to be supported by the recent identification of *ToxB*-like sequences in a number of other fungal species (Andrie *et al.*, 2008). Additional research is needed to identify and characterize these molecules and their mechanisms of action.

1.4. Proteomics and analysis tools

The proteome ('PROTEins expressed by the genOME') can be defined as the complete protein complement expressed by a genome, or by a cell or a tissue type (Wilkins *et al.*, 1995, 1996). The principal difference between the proteome and the genome is that the former can be changed in response to external or

internal stimuli, while the latter is unique among different types of organisms. The study of the proteome, or 'proteomics', can be defined as "the use of quantitative protein-level measurements of gene expression to characterize biological processes (e.g., disease processes and drug effects) and decipher the mechanisms of gene expression control" (Anderson and Anderson, 1998). Proteomics has become established as a powerful field of study and can provide information on the presence of protein isoforms, localization of gene products, post-translational modifications (such as phosphorylation and glycosylation), protein-protein interactions and the molecular composition of organelles (Pandy and Mann, 2000), while genomics and transcriptomics provide information on DNA sequences and gene expression. Overall, the goal of proteomics is complementary to that of genomics-based approaches, namely to elucidate and characterize the complete network of cell regulation.

Thanks to advances in biotechnological techniques over the past few decades, a number of methods are available to identify and analyze proteins. Two-dimensional gel electrophoresis (2-DE), a gel-based technique, was first described in 1975 by two independent researchers (Klose, 1975; O'Farrell, 1975). The technique combined two unrelated parameters (net charge and molecular mass) and enabled the separation of 1,100 different proteins from *Escherichia coli* (Migula) Castellani & Chalmers into individual components for the first time (O'Farrell, 1975). In the first-dimension step, isoelectric focusing (IEF) separates proteins according to their isoelectric points (pI) using immobilized pH gradient (IPG) strips containing carrier ampholytes, reducing agents and non-ionic or

zwitterionic detergents (Bjellqvist *et al.*, 1982; Görg *et al.*, 1988). In the second-dimension step, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their relative molecular mass (M_r) (O'Farrell, 1975). The most frequently and routinely used buffer system for SDS-PAGE in 2-DE is the Laemmli (1970) buffer system (tris-glycine system). This is a discontinuous buffer system that contains SDS in the buffer. When proteins are denatured by heating in the presence of a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol (βME), SDS binds to the proteins, masking their charges and resulting in the formation of SDS-protein complexes (Berkelman and Stenstedt, 1998). At this time, the SDS-protein structures become differentially negatively charged in a manner proportional to their molecular mass. The proteins can then be separated according to their M_r at high pH with different buffer ions in the gel and electrode reservoir (Laemmli, 1970).

Separated proteins can be visualized with a number of staining methods, including dyes or metals such as Coomassie Brilliant Blue, SYPRO, colloidal Coomassie Blue or silver stains. Recent advances in MS techniques, such as the high-throughput capability of MS using electrospray ionization (ESI) and MALDI, have made it possible to identify proteins separated by 2-DE, largely replacing traditional Edman degradation (Edman and Begg, 1967) for N-terminal sequencing. Prior to proceeding to MS analysis, however, proteins spots excised from gels need to be digested with a protease such as trypsin to generate peptides for exact protein identification (Pandy and Mann, 2000).

More than 20 phytopathogenic fungal species have now been studied using proteomics approaches. These studies have been undertaken to better understand a variety of processes, including plant-fungus interactions, fungal pathogenicity, virulence, life cycles and infection mechanisms (see reviews by Kav *et al.*, 2007 and Gonzalez-Fernandez and Jorrin-Novo, 2012). Among plant pathogenic fungi, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea* Pers. Fr., and *Fusarium graminearum* Schwabe have been the most studied species so far, as a consequence of their importance as agricultural pests (Kav *et al.*, 2007; Gonzalez-Fernandez and Jorrin-Novo, 2012). The mycelial proteome, secretome (and in the case of *S. sclerotiorum*, the proteomes of the sclerotia and sclerotial exudates) have all been analyzed in various independent studies.

Recently, a proteomics approach was taken to investigate the wheat response to the necrotrophic effector SnToxA produced by *S. nodorum* (Vincent *et al.*, 2012). Using 2-DE combined with MALDI-TOF/TOF MS/MS analysis, 91 differentially abundant proteins were identified, relative to a control treatment, in SnToxA-treated wheat leaves over a 72-h time-course. Most of these proteins were involved in PSI, PSII, the Calvin cycle, oxidative stress and defense responses, and many of them were targeted to the chloroplast. The results indicate that SnToxA causes a disruption in photosynthesis, resulting in the rapid accumulation of chloroplastic ROS, promoting cell death and pathogen growth. In this thesis, I have employed a proteomics approach, in combination with molecular biology and classical phytopathology techniques, to meet the research objectives outlined below.

1.5. Research objectives

The present study was undertaken to gain insights into the mode of action of Ptr ToxB and the role of this protein in fungal pathogenicity and virulence. To this end, three specific objectives were identified: (1) to examine the direct effects of Ptr ToxB on photosynthesis and the host leaf proteome at various time-points prior to the development of visible chlorosis symptoms, (2) to investigate the role of Ptr ToxB as both a pathogenicity and virulence factor via transformation of a Ptr ToxB non-producing isolate of *P. tritici-repentis* with the *ToxB* gene, and (3) to determine whether the acquisition of Ptr ToxB-producing ability can make a non-wheat pathogen (*P. teres*) infectious on wheat. Collectively, the work is intended not only to further our understanding of the role of Ptr ToxB in the tan spot pathosystem, but also to provide clues as to the possible role(s) of *ToxB* homologs in closely related species.

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2. Inhibition of photosynthesis and modification of the wheat leaf proteome by Ptr ToxB, a host-specific toxin from the fungal pathogen *Pyrenophora tritici-repentis**

2.1. Introduction

Tan or yellow leaf spot, caused by the ascomycete 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, causing yield losses of up to 50% (De Wolf *et al.*, 1998; Hosford, 1982). These losses result mainly from the development of necrotic and/or chlorotic lesions on infected leaves, which can significantly reduce total photosynthetic area. The necrosis and chlorosis symptoms are induced by the action of at least three host-specific toxins (HSTs), Ptr ToxA, Ptr ToxB and Ptr ToxC, which are differentially produced by the eight known races of *P. tritici-repentis* (Strelkov and Lamari, 2003). The Ptr toxins are generally regarded as pathogenicity factors, *sensu* Yoder (1980), and mediate compatible interactions between susceptible host genotypes and individual races of the pathogen.

Ptr ToxA [synonyms: Ptr necrosis toxin, Ptr toxin and ToxA (Ciuffetti *et al.*, 1998)] is a 13.2 kDa proteinaceous toxin that causes necrosis on susceptible

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wheat genotypes (Ballance *et al.*, 1989; Tomás *et al.*, 1990; Tuori *et al.*, 1995; Zhang *et al.*, 1997). This HST, which was the first to be characterized from *P. tritici-repentis*, is encoded by the single-copy *ToxA* gene (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). A second proteinaceous toxin, Ptr ToxB (synonym: Ptr chlorosis toxin), is 6.6 kDa in mass and induces chlorosis on susceptible hosts (Strelkov *et al.*, 1999). Ptr ToxB is encoded by a multi-copy gene termed *ToxB*, homologs of which are also found in isolates of *P. tritici-repentis* that do not possess any detectable Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006), as well as in *Pyrenophora bromi* and other ascomycete fungi (Andrie *et al.*, 2008). A third HST, Ptr ToxC, also causes chlorosis, but on different wheat lines/cultivars than Ptr ToxB (Gamba *et al.*, 1998). Unlike Ptr ToxA and Ptr ToxB, Ptr ToxC appears to be a low molecular mass, polar, non-ionic compound (Effertz *et al.*, 2002).

We previously suggested that the development of chlorosis in response to Ptr ToxB results from an inhibition of photosynthesis in the host, leading to the photooxidation of chlorophyll molecules as illuminated thylakoid membranes become unable to dissipate excess excitation energy (Strelkov *et al.*, 1998). Support for this hypothesis came from experiments with partially purified toxin, which revealed that: (1) the toxin has no effect on the greening of etiolated tissue, suggesting that it does not inhibit chlorophyll synthesis, (2) chlorosis is light-dependent and can be prevented by the application of p-benzoquinone, a compound that quenches triplet chlorophyll and singlet oxygen, and (3) chlorophyll degradation profiles obtained via high performance liquid

chromatography (HPLC) are consistent with photooxidation of the pigment (Strelkov *et al.*, 1998). The effect of Ptr ToxB on photosynthesis, however, was not directly evaluated, and to our knowledge, no other studies have examined the effects of this toxin on sensitive wheat leaves. As such, the mode of action of Ptr ToxB remains poorly understood.

In order to improve this understanding, we undertook a detailed analysis of Ptr ToxB-induced changes in the wheat leaf proteome, at various times prior to the development of visible chlorosis symptoms. We show that treatment with Ptr ToxB results in significant changes in the abundance of more than 100 proteins, including proteins involved in the light reactions of photosynthesis, the Calvin cycle, and the stress/defense response. The direct effect of Ptr ToxB on photosynthesis was also examined, with net photosynthesis found to decline within 12 h of toxin-treatment, long before chlorosis develops at 48 to 72 h. These results suggest that Ptr ToxB induces rapid and profound changes in toxin-sensitive wheat leaves, which precede and likely contribute to the development of the typical chlorosis symptoms. To our knowledge, this is the first proteomic analysis of the effects of Ptr ToxB on wheat. The production of host-specific toxins by *P. tritici-repentis*, which serve as pathogenicity factors for the fungus, makes tan spot an ideal pathosystem 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.

2.2. Materials and methods

2.2.1. Plant material

The wheat cultivar Katepwa (sensitive to Ptr ToxB) was used throughout this study. Plants were grown in 12.7 cm diameter plastic pots filled with Metro Mix® 220 potting medium (Grace Horticultural products, Ajax, ON, Canada), at a density of five 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.

2.2.2. Ptr ToxB production and treatment of host leaf tissue

Hexahistidine (His)-tagged Ptr ToxB was heterologously expressed in *Escherichia coli* and purified by affinity chromatography as described by Kim and Strelkov (2007). A 1 µM solution of the purified His-tagged toxin in 20 mM sodium acetate buffer, pH 4.6, was infiltrated with a Hagborg (1970) device into the second and third leaves of wheat seedlings at the 3-4 leaf stage. Controls were infiltrated only with the buffer solution. Tests were repeated three times, with two replicates consisting of two pots each included per treatment per time-point in each repetition of the experiment.

2.2.3. Effect of Ptr ToxB on photosynthesis

Net rates of photosynthesis were measured every 12 h for 6 days after treatment of leaves with Ptr ToxB or buffer, using an S151® Infrared Gas Analyzer (Qubit Systems, Kingston, ON, Canada) supplied with scrubbed moisture-free ambient CO_2 (350 ± 30 ppm) at 20ºC. Measurements were made on

the second leaf of each of three seedlings, with the infiltrated regions of the leaves placed together in the leaf chamber of the unit. The CO_2 exchange rate was recorded using a Lab Pro® interface and Logger Pro® 3 version software (Vernier Software & Technology, Beaverton, OR, USA), at a light intensity of 180 µmol photons m^{-2} s^{-1}. Photosynthetic rates were calculated as per the manufacturer's instructions (Qubit Systems).

2.2.4. Sample preparation and protein extraction

Leaf segments (total mass 300 mg), randomly chosen 12, 24 and 36 h after infiltration with Ptr ToxB or buffer, were cut, weighed and flash frozen in liquid nitrogen. Proteins from each treatment were extracted as described by Sharma *et al.* (2007). Briefly, pooled leaf tissue was ground to a fine powder in a mortar with a pestle in the presence of liquid nitrogen. The homogenate was transferred to a pre-chilled microcentrifuge tube and resuspended in 1.5 mL of ice-cold acetone containing 10% (w/v) trichloroacetic acid (TCA; Fisher Scientific Ltd., Ottawa, ON, Canada) and 0.07% (w/v) DTT (Fisher). After agitation with a vortex, 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, vigorously vortexed, and incubated again at –20°C for 1 h. The samples were centrifuged as above and the washing step repeated an additional four times. The washed pellets were vacuum-dried at room temperature (RT) until the acetone evaporated completely (Vacufuge Concentrator Model 5301, Eppendorf, Mississauga, ON). The dried protein pellets were dissolved in 500 µL of

rehydration/sample buffer (RB) (catalogue no. 163-2106, 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. Protein concentrations in the supernatants were determined according to the method of Bradford (1976), using the Protein Assay Dye Reagent (Bio-Rad); serial dilutions of BSA (Pierce Biotechnology Inc., Rockford, IL, USA) in RB containing TBP were used as standards. Proteins from buffer and Ptr ToxB-treated leaves were extracted separately, and samples were stored at –20°C until needed.

2.2.5. Two-dimensional electrophoresis

Isoelectric focusing (IEF) of extracted proteins in the first dimension and separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension were performed as described by Sharma *et al.* (2007). Briefly, 500 µg of each protein extract, in a total volume of 300 µL RB containing 2 mM TBP, were passively rehydrated overnight into 17 cm IPG strips (pH 4-7, linear, Bio-Rad), and IEF was conducted in a PROTEAN® IEF cell (Bio-Rad). After IEF, the IPG strips were equilibrated twice in 5 mL equilibration buffer 1 [6 M 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. Following saturation with equilibration buffer 1, the strips were incubated twice in equilibration buffer 2 [6 M 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. SDS-PAGE was carried out in a PROTEAN® II xi cell

(Bio-Rad) using the buffer system of Laemmli (1970) on 13% polyacrylamide gels at a constant voltage (90 V). Gels were stained using a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and destained overnight in distilled water.

2.2.6. Image analysis and protein identification

Stained 2-D gels were scanned on a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed using PDQuest software (Version 7.3.1, Bio-Rad). A set of 12 gel images was analyzed for each time-point, representing two gels each from the toxin-infiltrated and control treatments, for each of the three independent replications of the experiment. Protein spots were automatically detected and matched using PDQuest software (Bio-Rad), followed by manual validation to eliminate false, mismatched and unmatched spots. Protein spots that showed a statistically significant ($p<0.05$) difference in intensity in toxin-treated versus control tissues were identified using the Student's *t*-test feature of the PDQuest software (Bio-Rad). Fold-changes in the spot intensities between treatments were calculated and spots showing reproducible changes (up or down-regulation) were carefully excised from the gels with a sterile scalpel. The excised spots were subjected to ESI-quadrupole-TOF MS/MS (ESI-q-TOF MS/MS) for protein identification at the Institute for Biomolecular Design, University of Alberta (Edmonton, AB), using a q-TOF 2 mass spectrometer (Micromass, Manchester, UK) as described by Yajima and Kav (2006). The raw MS/MS data were converted to MASCOT generic files and used to search the entire National Center for Biotechnology Information non-redundant (NCBInr) database (downloaded 22

Sept. 2007) with the MASCOT online search engine (www.matrixscience.com). The search parameters included fixed modification of carbamidomethyl (C), one missed trypsin cleavage, possible oxidation of methionine, peptide mass tolerance of ± 0.1 Da, and fragment mass tolerance of ± 0.6 Da. Identified proteins were grouped into functional categories based on the classifications of the Munich Information Center for Protein Sequences (MIPS) (Ruepp *et al.*, 2004).

2.2.7. Enzyme assays

An assay for total peroxidase (POD) activity was conducted as described by Liang *et al.* (2008), while ascorbate peroxidase (APX) activity was measured as per Amako *et al.* (1994) with minor modifications. Briefly, leaf tissue (100 mg) was flash-frozen and ground to a fine powder in liquid N_2 with a mortar and pestle, followed by extraction in 1 mL of extraction buffer (50 mM potassium phosphate, pH 7.0, 1 mM ascorbic acid, and 1 mM EDTA). The homogenate was transferred to a pre-chilled microcentrifuge tube and centrifuged at 16,000 × g for 15 min at 4°C. The supernatant was collected and a 10 µL aliquot added to 180 µL of a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0) and 0.5 mM ascorbic acid. The enzymatic reaction was started by adding 10 µL of a 0.1 mM hydrogen peroxide solution to this mixture, and the rate of ascorbic acid oxidation was measured by monitoring the decrease in absorbance at 290 nm for a 30 s period after addition of the substrate (Amako *et al.*, 1994). Relative enzyme activities were calculated by dividing the specific activity of each enzyme (units mg^{-1} protein, where one unit is the amount of enzyme catalyzing the conversion of one µmole of hydrogen peroxide per minute) in the toxin-treated tissue by the

specific activity in the control treatment. Protein concentrations were measured as described above; all enzyme assays were repeated twice with wheat tissue protein extracts from three independent biological replicates.

2.2.8. Western blotting

Rabbit polyclonal antibodies specific for oxygen-evolving enhancer protein 1 (OEE1) and the beta subunit of ATP synthase (mitochondrial and chloroplast forms) were purchased from Abcam Inc. (Cambridge, MA), while polyclonal antibodies for germin-like protein (GLP) 1 were raised in rabbits at the Biosciences Animal Service Unit, University of Alberta. The GLP 1 antigen was prepared by protein electroelution as previously described (Leppard *et al.*, 1983) with some minor modifications. Briefly, GLP spots were excised with a sterile scalpel from 25 large 2-D gels (16 × 16 cm) that had been prepared and run as above, pooled, and placed in 1,000 molecular mass cut-off dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA) in 4.5 mL of 0.2 M Tris/acetate, pH 7.4, 1% SDS, and 100 mM DTT. The dialysis tubing was then transferred to a horizontal electrophoresis tank filled with 50 mM Tris/acetate, pH 7.4, and 0.1% SDS, and the proteins were electroeluted at 100 V at 4°C for 4 h. After removing the gel pieces from the dialysis tubing, the protein solution was dialyzed against distilled water overnight at 4°C with gentle stirring. The samples were lyophilized, re-suspended in sterile distilled water, and stored at –20°C until needed.

For Western blotting analysis, leaf protein extracts were extracted as described above, except that the dried protein pellets were dissolved in denaturing

buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea, and 20 mM βME, pH 8.0) instead of RB. The resulting suspensions were incubated at RT with stirring for 3 h and total soluble proteins were collected by centrifugation at 16,000 × g for 15 min. The proteins (10 µg total protein per sample) were separated by SDS-PAGE (Laemmli, 1970) and transferred to a PVDF membrane (BioRad) as per the procedures of Bjeruum and Schafer-Nielsen (1986). Western blotting analysis was then conducted as described by Cao *et al.* (2009). Blot images were scanned with a GS-800 Calibrated Densitometer (Bio-Rad) and band intensities were measured with Adobe Photoshop® CS2, version 9.0.2 (Adobe, San Jose, CA). Western blotting experiments were repeated twice for each time-point in each of three independent biological replicates.

2.2.9. N-terminal sequence analysis

Following 2-DE, proteins were transferred to a PVDF membrane (BioRad) as above, fixed/stained for 30 min in a solution of methanol-acetic acid-water (5:1:4, v/v/v) containing 0.05% (w/v) Coomassie Brilliant Blue R-250, and destained in methanol-acetic acid-water (0.5:0.7:8.8, v/v/v). The three spots corresponding to GLP, as determined by ESI-Q-TOF MS/MS, were cut from the PVDF membrane and sequenced at the Laboratory of Structure and Metabolism of Neuropeptides, Institut de Recherches Cliniques, Montréal, Québec, on an Applied Biosystems Procise® cLC-490 equipped with a phenylthiohydantoin (PTH) analyser (Applied Biosystems Inc., Foster City, CA). The PTH amino acid derivatives generated after each cycle of Edman degradation were identified by comparison with authentic standards (Hewick *et al.*, 1981). Identities of proteins

were established by searching the NCBI database with the deduced N-terminal amino acid sequence using the "Blastp" tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Results

2.3.1. Symptom development

As expected, chlorosis symptoms on the Ptr ToxB-infiltrated wheat leaves begun to develop at about 48 h after treatment with the toxin. By 72 h, the chlorosis had become very strong and spread throughout the infiltration zones. The centers of the infiltrated sites started to turn necrotic at 92 h, an observation consistent with previous reports (Kim and Strelkov, 2007; Strelkov *et al.*, 1999). No chlorosis developed on leaves infiltrated only with buffer, which remained green throughout the study.

2.3.2. Effect of Ptr ToxB on photosynthesis

Treatment with Ptr ToxB had a profound effect on photosynthesis, with the rate of foliar CO_2 uptake beginning to decline significantly within 12 h after infiltration with the toxin (Fig. 2-1). In contrast, the photosynthetic rate in leaves treated with buffer alone remained constant throughout the experiment. By 24 h after infiltration, net photosynthesis in the toxin-treated leaves had declined to 78% that of the controls, and by 36 h, at which time chlorosis was still not visible, it had declined to 67%. This decrease in the photosynthetic rate continued throughout the time-course of the study, so that by the time strong chlorosis developed at 72 h, net photosynthesis was only 2.57 μmol CO_2 m^{-2} s^{-1} in the Ptr

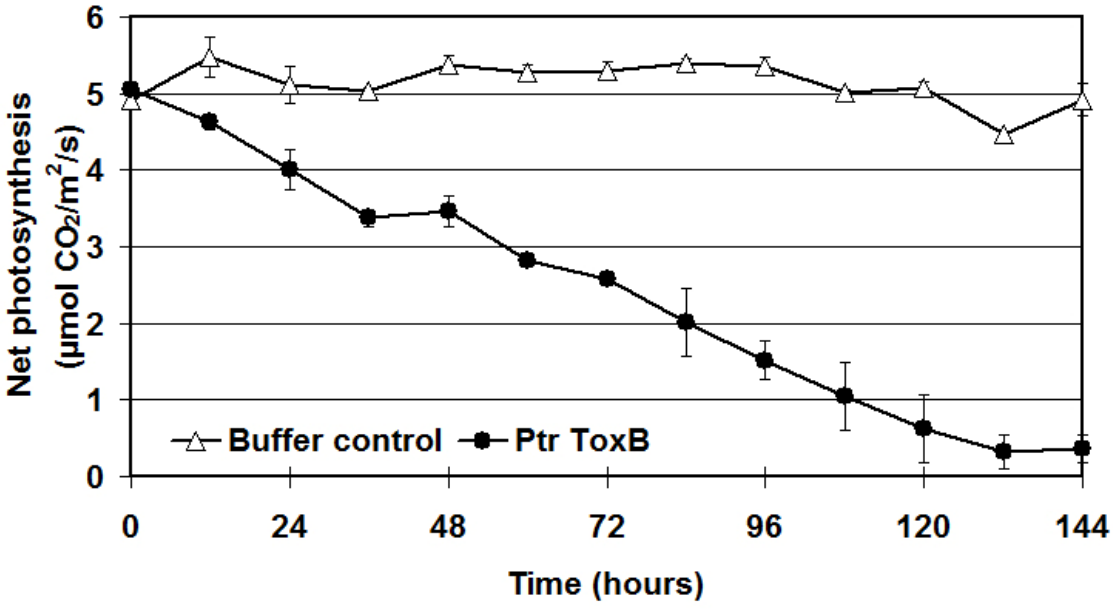


Figure 2-1. Net photosynthesis in the leaves of the toxin-sensitive wheat cv. Katepwa at various times after infiltration with 1 µM Ptr ToxB in 20 mM sodium acetate buffer, pH 4.6 (●), or with buffer alone (control) (Δ). The plants were maintained in a growth cabinet at 21/18°C (day/night) with a 16 h photoperiod at a light intensity of 180 µmol photons m^{-2} s^{-1}. Measurements of photosynthesis were taken every 12 h with an infrared gas analyzer (Qubit Systems, Kingston, ON) at 20°C and ambient CO_2 concentration (350 ± 30 ppm) at a light intensity of 180 µmol photons m^{-2} s^{-1}. The means from three biological replications of the experiment are shown; bars indicate the standard error of the mean.

ToxB-treated leaves, or 48% of the controls. By 120 h, the photosynthetic rate in the toxin-treatment had declined to about 12% of the controls.

2.3.3. Leaf proteome of wheat treated with Ptr ToxB

Analysis of the leaf proteome by 2-DE (Fig. 2-2) revealed an average (± S.E.) of 318 ± 0, 288 ± 0 and 461± 0 total spots for leaf tissue collected at 12, 24 and 36 h after toxin treatment, respectively. A total of 102 protein spots were reproducibly and significantly ($p<0.05$) altered in intensity between toxin-treated and control tissues, of which 66 were more abundant and 36 were less abundant after treatment with Ptr ToxB. All of the differentially abundant proteins were subjected to ESI-Q-TOF MS/MS, and the identities of 47 of these proteins were established through a database search (Table 2-1). Highly significant ($p<0.0001$) correlations were found between the predicted and observed molecular masses for all of the identified proteins. Similarly, significant ($p<0.01$) correlations were also observed between the predicted and observed p*I* values for most (94%) of the differentially abundant proteins. In the few cases for which a significant correlation was not found, the predicted p*I* values were greater than 8 (Table 2-1); thus, the discrepancies may have resulted from the pH range used in the IEF step (pH 4 to 7). The detection of some spots corresponding to the same protein probably reflected the occurrence of post-translational modifications and/or different isoforms of these proteins (Devos *et al.*, 2006). The differentially abundant proteins could be grouped into at least seven MIPS functional categories (FunCat) (Fig. 2-3).

Figure 2-2. Changes in the leaf proteome of wheat cv. Katepwa induced by treatment with Ptr ToxB. (A) Representative 2-D gel image of proteins extracted from the leaf tissue and stained with Colloidal Coomassie Blue. Protein spots exhibiting significant ($p<0.05$) differences in intensity and identified by MS/MS are indicated by arrows and numbers, and are listed in Table 2-1. (B) A closer view of the spots showing significantly different intensities between toxin-infiltrated (T) and control (C) treatments, where arrows highlight spots of interest when these are not obvious. Spots 1 to 12 represent differentially abundant proteins identified at 12 h, spots 13 to 38 represent differentially abundant proteins at 24 h, and spots 39 to 47 represent differentially abundant proteins at 36 h.

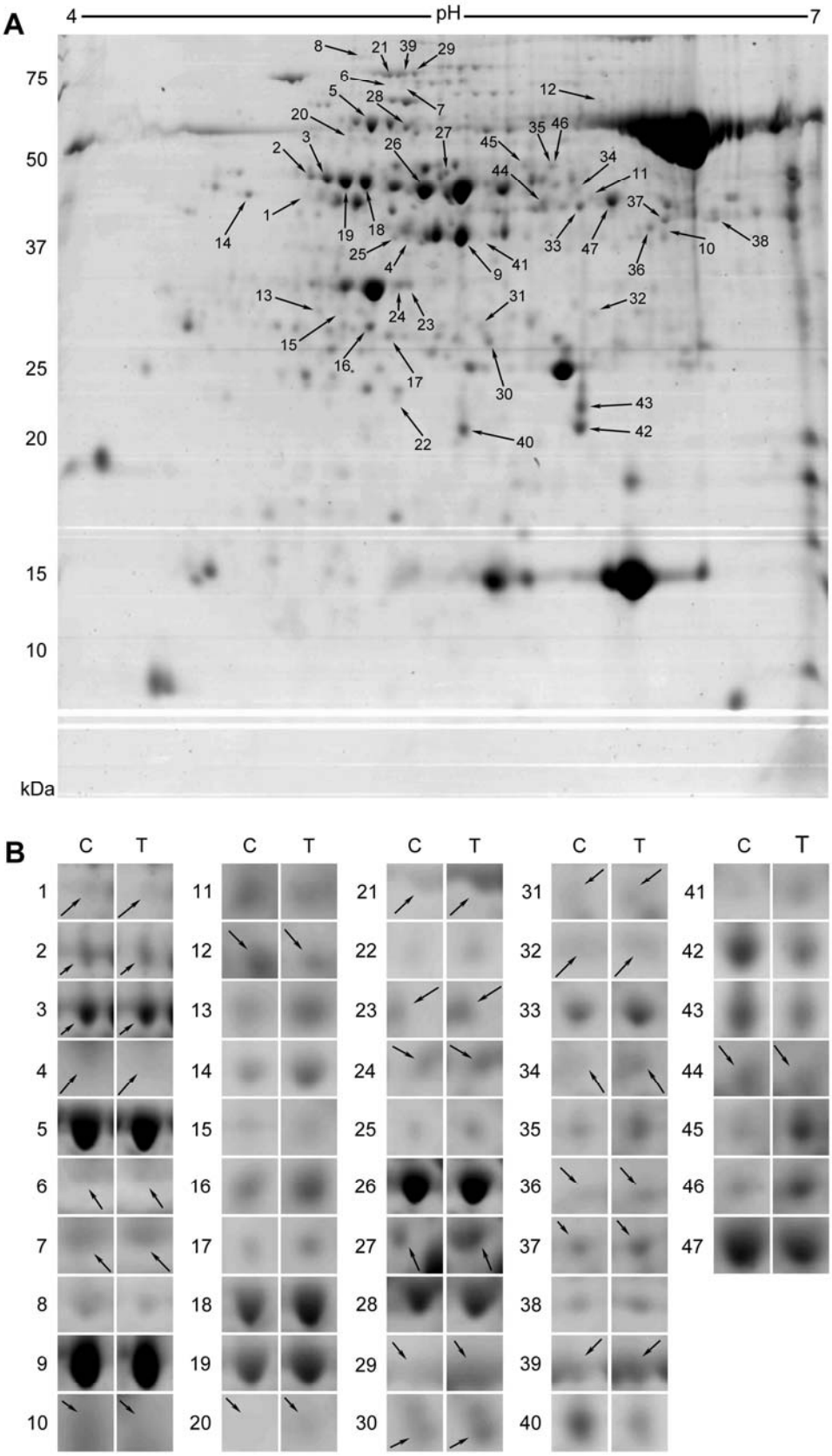

A
4
pH
7
75
50
37
25
20
15
10
kDa
B
C
T

Table 2-1. Details of proteins found to be differentially abundant in leaves of wheat cv. Katepwa 12, 24 and 36 h after infiltration with Ptr ToxB.

Spot	Time (h)	Protein identity[a]	MS/MS ESI-Q-ToF			Access ID[d]	MW (kDa/p*I*)		Fold change[e]
			PM/%[b]	Score[c]	Sequence		Theor.	Obs.	
1	12	Phosphoribulokinase, chloroplast precursor (Phosphopentokinase) (PRKase) (PRK) [*Triticum aestivum*]	2/5%	58 (>54)	R.KPDFDAFIDPQK.Q R.DLYEQIIAER.A	gi|125580	45.5/ 5.72	43.2/ 4.91	2.01 ± 0.29 ↓
2	12	Plastid glutamine synthetase isoform GS2c [*Triticum aestivum*]	3/9%	179 (>54)	K.AILNLSLR.H R.HDLHIAAYGEGNER.R R.LTGLHETASISDFSWGVANR.G	gi|71362640	47.0/ 5.75	47.4/ 5.01	2.05 ± 0.28 ↓
3	12	Plastid glutamine synthetase isoform GS2b [*Triticum aestivum*]	3/9%	237 (>54)	K.AILNLSLR.H R.HDLHIAAYGEGNER.R R.LTGLHETASISDFSWGVANR.G	gi|71362638	46.9/ 6.04	47.1/ 5.09	1.41 ± 0.06 ↓
4	12	Fructose-bisphosphate aldolase class-I [*Oryza sativa* (japonica cultivar-group)]	2/6%	77 (>54)	R.LASIGLENTEANR.Q R.EAAYYQQGAR.F	gi|62732954	39.5/ 6.85	36.9/ 5.38	3.37 ± 0.96 ↓
5	12	ATP synthase CF1 beta subunit [*Triticum aestivum*]	5/18%	482 (>54)	R.DTDDKQINVTCEVQQLLGNNR.V R.GMEVIDTGAPLSVPVGGATLGR.I K.AHGGVSVFGGVGER.T R.DVNKQDVLLFIDNIFR.F K.GIYPAVDPLDSTSTMLQPR.I	gi|14017579	53.8/ 5.06	54.3/ 5.23	1.59 ± 0.52 ↓
6	12	Putative FtsH-like protein Pftf precursor [*Oryza sativa* (japonica cultivar-group)]	1/2%	62 (>55)	R.AILSEFTEIPVENR.V	gi|52075838	72.6/ 5.54	69.3/ 5.28	3.27 ± 1.14 ↓
7	12	Putative FtsH-like protein Pftf precursor [*Oryza sativa* (japonica cultivar-group)]	3/5%	154 (>55)	R.FLEYLDKDR.V R.ADILDSALLRPGR.F R.AILSEFTEIPVENR.V	gi|52075838	72.6/ 5.54	70.9/ 5.35	2.95 ± 0.78 ↓
8	12	OSJNBa0091D06.15 [*Oryza sativa* (japonica cultivar-group)]	3/6%	120 (>54)	K.LAQEDPSFHFSR.D R.VEANVGAPQVNYR.E K.VEVITPEEHLGDVIGDLNSR.R	gi|38567873	82.4/ 5.69	78.9/ 5.18	8.15 ± 4.60 ↓

9	12	Fructose-bisphosphate aldolase class-I [*Oryza sativa* (japonica cultivar-group)]	1/2%	57 (>42)	R.EAAYYQQGAR.F	gi|62732954	39.5/ 6.85	38.7/ 5.58	1.24 ± 0.10 ↓
10	12	Ferredoxin-NADP(H) oxidoreductase [*Triticum aestivum*]	1/4%	97 (>54)	R.LYSIASSALGDFGDAK.T	gi|20302471	39.1/ 8.29	38.1/ 6.39	1.66 ± 0.22 ↓
11	12	ATP synthase subunit gamma, chloroplast precursor [Contains: Inceptin] [*Zea mays*]	3/6%	77 (>54)	K.VALVVLTGER.G K.VALVVLTGER.G R.ALQESLASELAAR.M	gi|110278822	40.1/ 8.44	42.2/ 6.18	5.15 ± 1.65 ↓
12	12	ATP synthase CF1 alpha subunit [*Triticum aestivum*]	2/5%	165 (>54)	R.IAQIPVSEAYLGR.V R.EAYPGDVFYLHSR.L	gi|14017569	55.3/ 6.11	61.2/ 6.12	2.34 ± 0.43 ↓
13	24	Harpin binding protein 1 [*Triticum aestivum*]	2/9%	89 (>54)	R.LLPITLGQVFQR.I K.TNGNLSQLPLLEVPR.I	gi|38679333	29.0/ 7.77	31.1/ 5.02	3.70 ± 1.86 ↑
14	24	Os08g0382400 [*Oryza sativa* (japonica cultivar-group)]	2/10%	80 (>54)	R.IVLDGYNAPVTAGNFLDLVER.K R.ADGFVVQTGDPEGPAEGFIDPSTGK.V	gi|115476198	46.7/ 4.82	42.4/ 4.74	1.42 ± 0.22 ↑
15	24	Harpin binding protein 1 [*Triticum aestivum*]	2/9%	173 (>54)	R.LLPITLGQVFQR.I K.GNLSQLPLLEVPR.I	gi|38679331	29.5/ 9.51	29.4/ 5.11	1.44 ± 0.27 ↑
16	24	Ascorbate peroxidase [*Hordeum vulgare*]	2/8%	196 (>55)	R.QDKPEPPPEGR.L R.LPDATQGSDHLR.Q	gi|15808779	27.9/ 5.10	28.1/ 5.23	2.32 ± 0.54 ↑
17	24	Cytosolic ascorbate peroxidase [*Zea mays*]	2/9%	134 (>55)	R.QDKPEPPPEGR.L R.LPDATQGSDHLR.Q	gi|600116	27.4/ 5.28	27.7/ 5.31	1.47 ± 0.23 ↑
18	24	Plastid glutamine synthetase isoform GS2c [*Triticum aestivum*]	2/5%	144 (>55)	K.AILNLSLR.H R.HDLHIAAYGEGNER.R	gi|71362640	47.0/ 5.75	47.1/ 5.13	1.60 ± 0.23 ↑
19	24	Plastid glutamine synthetase isoform GS2a [*Triticum aestivum*]	2/5%	165 (>55)	K.AILNLSLR.H R.HDLHIAAYGEGNER.R	gi|71362455	47.0/ 5.42	46.8/ 5.21	1.35 ± 0.13 ↑

20	24	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplast precursor (RuBisCO activase A) (RA A) [*Hordeum vulgare*]	11/32%	98 (>80)	K.GLAYDISDDQQDITR.G K.NFMTLPNIKIPLILGIWGGK.G K.SFQCELVFAK.M K.MCCLFINDLDAGAGR.M R.VPIVVTGNDFSTLYAPLIR.D K.FYWAPTR.D K.GIFQTDNVSDESVVK.I K.IVDTFPGQSIDFFGALR.A R.VYDDEVRK.W R.DGPVTFEQPK.M K.LLEYGHMLVQEQDNVKR.V	gi|12643756	51.3/ 8.04	52.3/ 5.19	3.44 ± 0.61 ↑
21	24	HSP70 [*Triticum aestivum*]	4/7%	182 (>56)	R.VEIIANDQGNR.T R.TTPSYVAFTDTER.L R.MVNHFVQEFK.R K.STVHDVVLVGGSTR.I	gi|2827002	71.3/ 5.14	72.7/ 5.28	3.16 ± 1.54 ↑
22	24	Os12g0115900 [*Oryza sativa* (japonica cultivar-group)]	1/4%	69 (>55)	R.IIDFDLPITVR.L	gi|115487008	25.1/ 8.88	23.2/ 5.34	1.37 ± 0.17 ↑
23	24	Chloroplast oxygen-evolving enhancer protein 1 [*Leymus chinensis*]	3/10%	120 (>55)	K.RLTFDEIQSK.T R.VPFLFTVK.Q R.GGSTGYDNAVALPAGGR.G	gi|147945622	34.7/ 6.08	32.6/ 5.38	1.50 ± 0.28 ↑
24	24	Chloroplast oxygen-evolving enhancer protein 1 [*Leymus chinensis*]	3/12%	95 (>56)	K.DGIDYAAVTVQLPGGER.V R.VPFLFTVK.Q R.GGSTGYDNAVALPAGGR.G	gi|147945622	34.7/ 6.08	32.5/ 5.36	1.89 ± 0.44 ↑
25	24	Hypothetical protein OsJ_004859 [*Oryza sativa* (japonica cultivar-group)]	1/2%	98 (>56)	K.IQGIGAGFVPR.N	gi|125573519	43.9/ 6.05	38.4/ 5.33	1.91 ± 0.62 ↑
26	24	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase [*Pachysandra terminalis*]	3/11%	159 (>56)	K.SFQCELVFAK.M R.VPVIVTGNDFSTLYAPLIR.D K.FYWAPTR.D	gi|94549022	34.8/ 5.55	43.9/ 5.44	1.33 ± 0.12 ↑
27	24	Actin [*Setaria italica*]	4/13%	324 (>43)	K.IWHHTFYNELR.V R.VAPEEHPVLLTEAPLNPK.A R.GYSFTTTAER.E K.GEYDESGPAIVHR.K	gi|9965319	41.8/ 5.31	48.3/ 5.51	16.91 ± 11.24 ↑

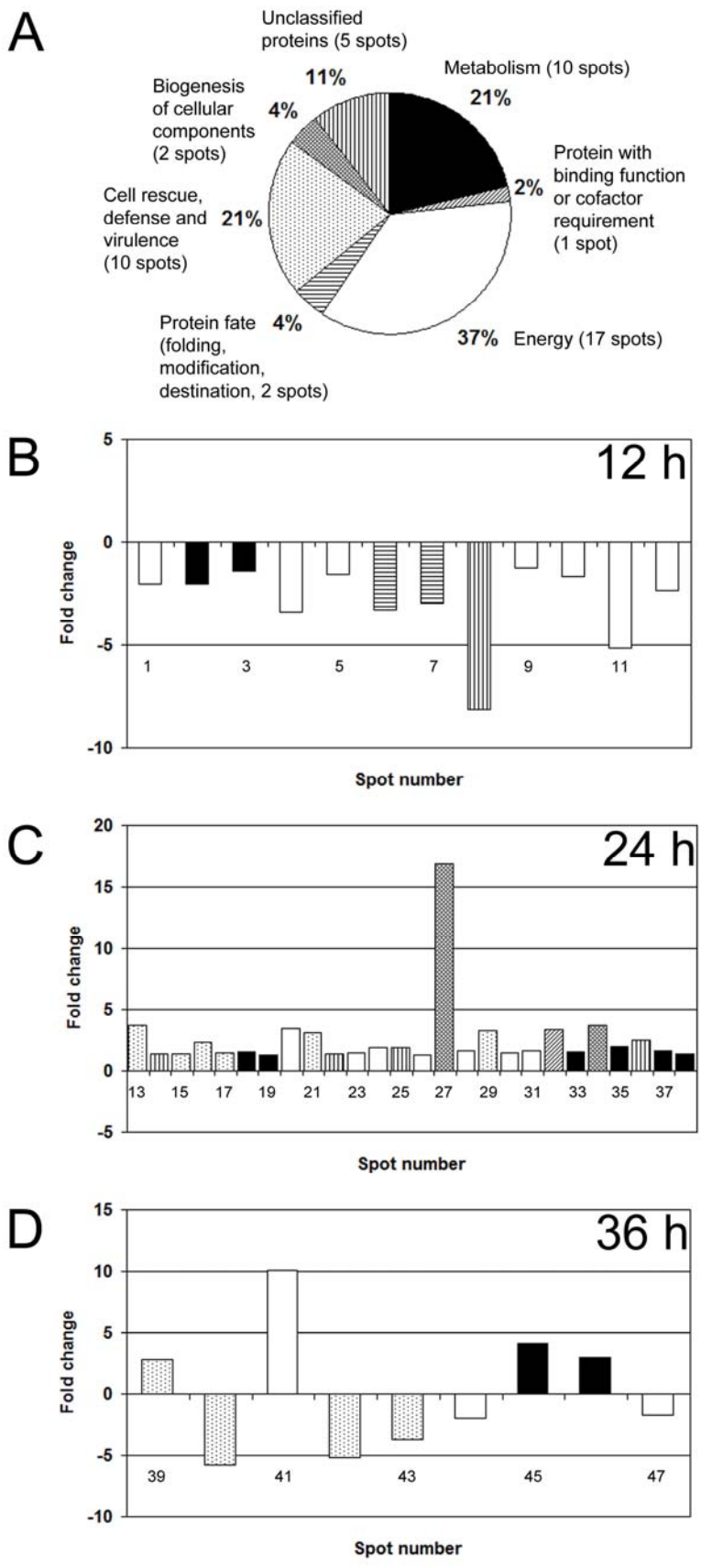
28	24	ATP synthase beta subunit [*Triticum aestivum*]	2/4%	122 (>56)	K.VVDLLAPYQR.G R.VGLTGLTVAEHFR.D	gi|525291	59.3/ 5.56	54.1/ 5.37	1.69 ± 0.34 ↑
29	24	HSP70 [*Hordeum vulgare* subsp. *vulgare*]	8/15%	113 (>80)	K.NGHVEIIANDQGNR.I R.ITPSWVGFTDGER.L K.NQAAVNPER.T K.VFSPEEVSAMILGK.M K.DAGVIAGLNVAR.I R.ALSNQHQVR.V R.FEELNNDLFR.K K.TQIHEIVLVGGSTR.I	gi|476003	67.1/ 5.76	72.9/ 5.41	3.27 ± 1.18 ↑
30	24	Triosephosphat-isomerase [*Triticum aestivum*]	2/9%	170 (>57)	R.SLMGESSEFVGEK.V K.VIACVGETLEQR.E	gi|11124572	27.0/ 5.38	27.4/ 5.68	1.52 ± 0.24 ↑
31	24	Triosephosphate isomerase, chloroplast precursor (TIM) (Triose-phosphate isomerase) [*Secale cereale*]	1/5%	58 (>55)	K.VASPEQAQEVHAAVR.D	gi|1174745	31.9/ 6.00	28.3/ 5.61	1.66 ± 0.34 ↑
32	24	Catalytic/ coenzyme binding [*Arabidopsis thaliana*]	1/5%	68 (>55)	R.KAEQYLADSGIPYTIIR.A	gi|18404496	34.9/ 8.37	31.5/ 6.13	3.36 ± 1.32 ↑
33	24	Cytosolic malate dehydrogenase [*Triticum aestivum*]	2/8%	84 (>54)	K.MELIDAAFPLLK.G K.AQASALEAHAAPNCK.V	gi|49343245	35.8/ 5.75	41.4/ 6.12	1.56 ± 0.31 ↑
34	24	Reversibly glycosylated polypeptide [*Triticum aestivum*]	1/3%	97 (>54)	K.VPEGFDYELYNR.N	gi|4158232	41.9/ 5.82	43.7/ 6.14	3.78 ± 0.77 ↑
35	24	S-adenosylmethionine synthetase 1 [*Triticum monococcum*]	4/12%	261 (>54)	R.NIGFISDDVGLDADR.C K.TIFHLNPSGR.F R.FVIGGPHGDAGLTGR.K K.TAAYGHFGR.E	gi|115589744	43.2/ 5.61	49.5/ 5.92	2.00 ± 0.37 ↑
36	24	Hypothetical protein OsJ_017648 [*Oryza sativa* (japonica cultivar-group)]	1/2%	69 (>54)	R.FVESAEAHFR.M	gi|125594106	40.4/ 6.90	38.3/ 6.14	2.54 ± 0.74 ↑
37	24	Putative NAD-malate dehydrogenase [*Oryza sativa* (japonica cultivar-group)]	1/3%	134 (>54)	K.KLFGVTTLDVVR.A	gi|42407501	41.7/ 7.01	40.7/ 6.38	1.64 ± 0.35 ↑

38	24	Putative NAD-malate dehydrogenase [*Oryza sativa* (japonica cultivar-group)]	1/3%	106 (>54)	K.KLFGVTTLDVVR.A	gi|42407501	41.7/ 7.01	40.9/ 6.57	1.43 ± 0.24 ↑
39	36	HSP70 [*Triticum aestivum*]	1/2%	43 (>42)	K.NAVVTVPAYFNDSQR.Q	gi|2827002	71.3/ 5.14	72.8/ 5.37	2.80 ± 0.57 ↑
40	36	Germin-like protein 1 [*Oryza sativa*]	2/10 %	140 (>54)	K.AAVTPAFVGQFPGVNGLGISAAR.L K.AAVTPAFVGQFPGVNGLGISAAR.L	gi|4239821	22.0/ 6.01	20.9/ 5.59	5.72 ± 0.96 ↓
		Adenosine diphosphate glucose pyrophosphatase [*Triticum aestivum*]	2/10%	140 (>54)		gi|21322655	21.9/ 5.68		
41	36	Fructose-bisphosphate aldolase, chloroplast precursor, putative, expressed *[Oryza sativa* (japonica cultivar-group)]	1/2%	66 (>54)	R.EAAYYQQGAR.F	gi|108864048	41.8/ 6.07	37.1/ 5.64	10.1 ± 5.1 ↑
42	36	Germin-like protein 1 [*Oryza sativa*]	2/10%	129 (54)	K.AAVTPAFVGQFPGVNGLGISAAR.L K.AAVTPAFVGQFPGVNGLGISAAR.L	gi|4239821	22.0/ 6.01	21.2/ 6.11	5.16 ± 1.06 ↓
		Adenosine diphosphate glucose pyrophosphatase [*Triticum aestivum*]	2/10%	129 (54)		gi|21322655	21.9/ 5.68		
43	36	Germin-like protein 1 [*Oryza sativa*]	2/15%	226 (>54)	K.AAVTPAFVGQFPGVNGLGISAAR.L K.VTFLDDAQVK.K	gi|4239821	22.0/ 6.01	22.9/ 6.11	3.71 ± 0.78 ↓
		Adenosine diphosphate glucose pyrophosphatase [*Triticum aestivum*]	2/15%	226 (>54)		gi|21322655	21.9/ 5.68		
		Germin-like protein [*Zea mays*]	2/15%	226 (>54)		gi|37623879	22.1/ 6.02		
		Germin-like protein 2a [*Hordeum vulgare* subsp. vulgare]	2/15%	226 (>54)		gi|109729537	21.9/ 5.68		

44	36	Ribulose 1,5-bisphosphate carboxylase activase isoform 1 [*Hordeum vulgare* subsp. *vulgare*]	12/41%	81 (>80)	K.WKGLAYDISDDQQDITR.G K.NFMTLPNIKIPLILGIWGGK.G K.SFQCELVFAK.M K.MCCLFINDLDAGAGR.M R.VPIVVTGNDFSTLYAPLIR.D K.FYWAPTR.D K.GIFQTDNVSDESVVK.I K.IVDTFPGQSIDFFGALR.A R.VYDDEVRK.W R.DGPVTFEQPK.M K.LLEYGHMLVQEQDNVKR.V R.VQLADTYMSQAALGDANQDAMK.T	gi|167096	47.3/ 8.62	41.5/ 5.89	1.97 ± 0.34 ↓
45	36	S-adenosylmethionine synthetase 1 [*Triticum monococcum*]	3/9%	218 (>54)	R.NIGFISDDVGLDADR.C R.FVIGGPHGDAGLTGR.K K.TAAYGHFGR.E	gi|115589744	43.2/ 5.61	49.8/ 5.82	4.09 ± 1.41 ↑
46	36	S-adenosylmethionine synthetase 1 [*Triticum monococcum*]	2/7%	122 (>54)	R.NIGFISDDVGLDADR.C R.FVIGGPHGDAGLTGR.K	gi|115589744	43.2/ 5.61	49.5/ 5.92	2.97 ± 0.65 ↑
47	36	Ribulose 1,5-bisphosphate carboxylase activase isoform 1 [*Hordeum vulgare* subsp. *vulgare*]	3/7%	130 (>54)	K.SFQCELVFAK.M K.FYWAPTR.D K.GIFQTDNVSDESVVK.I	gi|167096	47.3/ 8.62	41.9/ 5.18	1.67 ± 0.34 ↓

[a] Proteins were identified using ESI-q-TOF MS/MS data.
[b] Number of peptides matched/percent sequence coverage.
[c] Ion score is -10 log (*P*), where *P* is probability that the observed match is a random event; scores greater than the threshold values in parentheses indicate identity or extensive homology ($p<0.05$).
[d] Access ID is the access identification of the protein from the non-redundant database search.
[e] Fold-increases (↑) or decreases (↓) (± S.E.) refer to differences in spot intensities between leaves treated with 1 µM Ptr ToxB in 20 mM sodium acetate buffer, pH 4.6, and those treated with buffer alone.

Figure 2-3. (A) Functional classification of differentially abundant proteins identified by tandem mass spectrometry. The proteins were grouped into categories based on the classifications of the Munich Information Center for Protein Sequences. The number of protein spots within each class is indicated in parentheses after the name of the category. (B – D) Graphical representation of fold changes in abundance of individual protein spots at 12 h (B), 24 h (C) and 36 h (D) after treatment with 1 μM Ptr ToxB in 20 mM sodium acetate buffer, pH 4.6, relative to buffer-treated controls. The patterns of the bars in (B) to (D) correspond to the patterns in (A), and denote the functional classification of each spot. Spot details are given in Table 2-1.


A
Unclassified proteins (5 spots)
11%
Metabolism (10 spots)
21%
Biogenesis of cellular components (2 spots)
4%
Protein with binding function or cofactor requirement (1 spot)
2%
Cell rescue, defense and virulence (10 spots)
21%
Protein fate (folding, modification, destination, 2 spots)
4%
37% Energy (17 spots)
B
12 h
Fold change
Spot number
C
24 h
Fold change
Spot number
D
36 h
Fold change
Spot number

The largest functional class (37%) consisted of proteins with roles in energy metabolism (MIPS FunCat 02). Spot 1 (Fig. 2-2; Table 2-1) was identified as phosphoribulokinase (PRKase) chloroplast precursor, and was down-regulated two-fold at 12 h after toxin-treatment. Two spots (4 and 9) corresponding to fructose-bisphosphate aldolase (FBA) class I were also down-regulated at 12 h, while a spot (41) corresponding to FBA chloroplast precursor was up-regulated at 36 h. Triosephosphate-isomerase (TIM) (spot 30) and TIM chloroplast precursor (spot 31) were found to be significantly increased in abundance at 24 h after toxin-treatment. An ATP synthase CF1 beta subunit was down-regulated at 12 h (spot 5), as were an ATP synthase CF1 alpha subunit (spot 12) and an ATP synthase gamma subunit chloroplast precursor (spot 11). At 24 h, an ATP synthase beta subunit was up-regulated nearly two-fold (spot 28), although this appeared to be the mitochondrial form (Abulafia *et al.*, 1996). Spot 10, corresponding to ferredoxin-NADP(H) oxidoreductase (FNR), was also found to be down-regulated at 12 h after infiltration with the toxin. Spots matching ribulose bisphosphate carboxylase/oxygenase (RuBisCo) activase were up-regulated at 24 h (spots 20 and 26) and down-regulated at 36 h (spots 44 and 47). Spots 23 and 24, both of which corresponded to OEE1, were also up-regulated at 24 h.

Proteins involved in general metabolism (MIPS FunCat 01) and in cell rescue, defense and virulence (MIPS FunCat 32) constituted the next two largest groups of differentially abundant spots, with each class representing 21% of the total (Fig. 2-3). Among the proteins with roles in general metabolism were plastid

glutamine synthetase (GS) isoforms GS2c (spots 2 and 18), GS2b (spot 3), and GS2a (spot 19); spots 2 and 3 were down-regulated at 12 h, while spots 18 and 19 were up-regulated at 24 h. Spots 33, 37 and 38, which were identified as cytosolic or putative NAD-malate dehydrogenase (MDH), were found to be up-regulated at 24 h. Similarly, spots 35, 45 and 46, which corresponded to *S*-adenosylmethionine (AdoMet) synthetase 1, were up-regulated more than two and three-fold in response to Ptr ToxB at 24 h and 36 h, respectively. With respect to proteins involved in cell rescue, defense and virulence, two spots (13 and 15) identified as Harpin-binding protein 1 were found to be up-regulated at 24 h after toxin treatment. Two proteins (spots 16 and 17) corresponding to APX were also up-regulated at this time. However, spots 40, 42 and 43, identified as GLP 1, were down-regulated three and five-fold at 36 h after treatment with Ptr ToxB. Heat shock protein (HSP) 70 was observed to be up-regulated at both 24 h (spots 21 and 29) and 36 h (spot 39).

The remaining differentially abundant proteins belonged to at least four different functional classes, each represented by one to five spots. Two spots (6 and 7), which were identified as putative FtsH-like protein Pftf precursor and classified as having a role in protein fate (MIPS FunCat 14), were down-regulated at 12 h after toxin treatment. Another two spots corresponded to proteins involved in the biogenesis of cellular components (MIPS FunCat 42), including actin (spot 27) and reversibly glycosylated polypeptide (RGP) (spot 34), and were up-regulated 17-fold and 4-fold, respectively, at 24 h. Spot 32 was identified as catalytic/coenzyme binding protein, which has a binding function or co-factor

requirement (MIPS FunCat 16), and was up-regulated three-fold at 24 h. The remaining five spots (11% of the total) corresponded to hypothetical or unclassified proteins (MIPS FunCat 99); spot 8 was down-regulated at 12 h, while spots 14, 22, 25 and 36 were up-regulated at 24 h.

2.3.4. Ascorbate peroxidase and total peroxidase activity

Enzyme assays revealed that relative APX activity was significantly (p<0.05) increased in the Ptr ToxB-infiltrated leaf tissue, relative to the control tissue, at 24 h after treatment (Fig. 2-4A). This finding is consistent with our 2-DE results, which indicated that two spots (16 and 17) corresponding to APX were significantly up-regulated only at 24 h after infiltration with the toxin (Table 2-1). Total POD activity, however, was found to be significantly increased at all three time-points (12 h, 24 h, 36 h) of the experiment (Fig. 2-4B).

2.3.5. Partial amino acid sequence of GLP-matching spots

The identities of spots 40, 42, and 43, which corresponded to GLP 1 as determined by ESI-q-TOF MS/MS (Table 2-1), were validated by N-terminal amino acid sequence analysis. The sequences obtained were: LTQDFCVADLXCXDT for spot 40; XXXXXFCVADL for spot 42; and LTQDFCVADLXXXXT for spot 43. These *de novo* amino acid sequences matched perfectly the Box A (QDFCVAD) motif found in the N-termini of germins and GLPs (Bernier and Berna, 2001). Moreover, they bore a very high degree of homology (E-value 6×10^{-4}) to GLP and ADP glucose pyrophosphatase accessions in GenBank (data not shown), confirming their identities as GLPs.

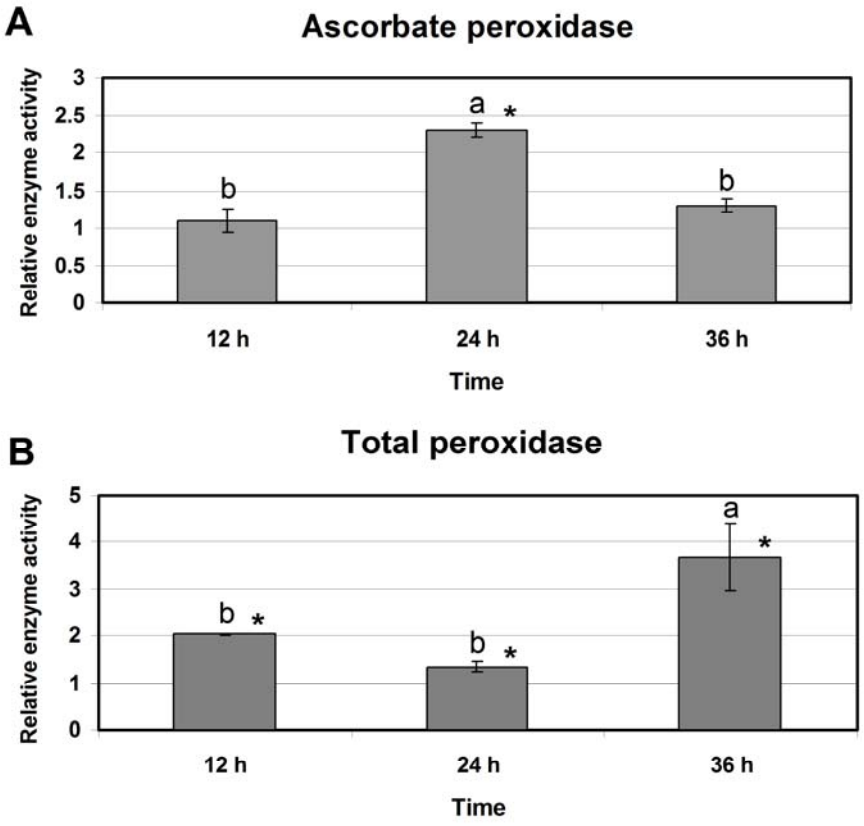


Figure 2-4. Ascorbate peroxidase (A) and total peroxidase (B) activity in leaves of wheat cv. Katepwa after infiltration with 1 μM Ptr ToxB in 20 mM sodium acetate buffer, pH 4.6, relative to activity in leaves infiltrated with buffer alone (control). The relative enzyme activities were calculated by dividing the specific activity of each enzyme (units mg^{-1} protein) in the toxin-treated tissue by the specific activity in the control treatment. Different letters indicate statistically significant ($p<0.05$) differences between toxin and control treatments over the entire time-course of the study, as determined by ANOVA. Asterisks (*) indicate significant ($p<0.05$) differences between toxin and control-treatments at individual time-points as per the Student's *t*-test. The means from three biological replications of the experiment are shown; bars indicate the standard error of the mean.

2.3.6. Western blotting

Polyclonal antibodies specific for the ATP synthase beta subunit reacted against one band approximately 55 kDa in size when these antibodies were incubated against total foliar proteins separated by 1-DE (Fig. 2-5A and 2-5B). The intensity of this band was 21.1 ± 0.6 % weaker in the Ptr ToxB-treated versus control tissue at 12 h after infiltration (Fig. 2-5A), while it was 20 ± 0.7 % stronger in the toxin-treated tissue at 24 h (Fig. 2-5B). Thus, the relative band intensities in the Western blots were consistent with the changes in the relative abundance of spots 5 and 28 (corresponding to the chloroplast and mitochondrial ATP synthase beta subunits, respectively) as found by 2-DE at 12 and 24 h, respectively (Table 2-1). Antibodies for OEE1 also reacted against a single band, approximately 33 kDa in size, when incubated against total wheat foliar proteins separated by 1-DE (Fig. 2-5C). The intensity of this band in the Ptr ToxB-treated tissue was increased by 23 ± 1.2 % relative to the control at 24 h after infiltration, a finding consistent with the increased abundance of spots matching OEE1 (spots 23 and 24) also observed at this time by 2-DE (Table 2-1).

Polyclonal antibodies raised against spots 40, 42 and 43 (corresponding to GLP 1, Table 2-1) reacted against two bands approximately 21 and 23 kDa in size when these antibodies were incubated against total foliar proteins separated by 1-DE (Fig. 2-5D). The observation that the three GLP spots identified in the 2-D gels were represented by two bands in the 1-D gels is likely due to the almost identical masses of spots 40 and 42 (Fig. 2A). Moreover, the Western blot analysis revealed a significant difference in band intensities between Ptr ToxB

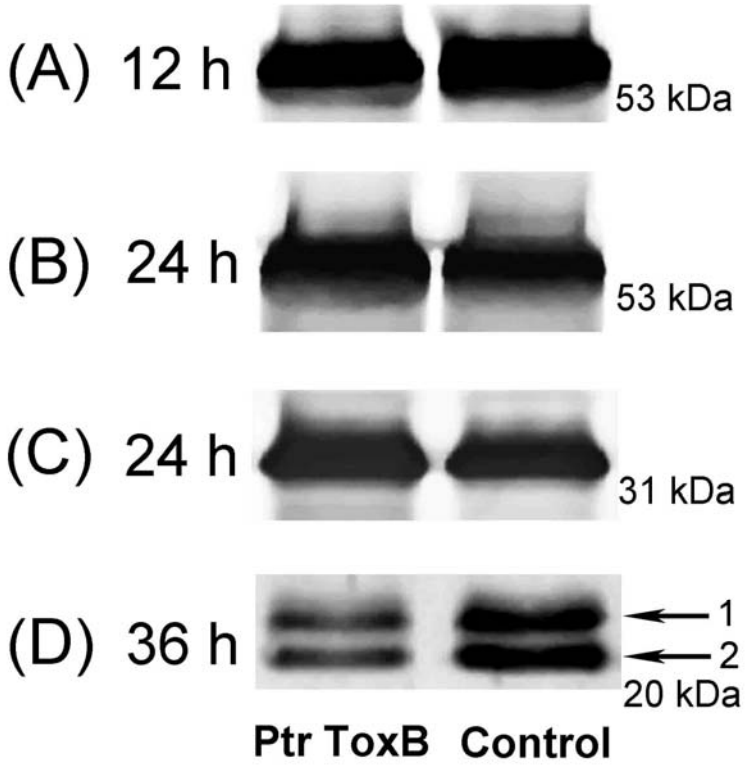


Figure 2-5. Western blot analysis of protein extracts from leaves of wheat cv. Katepwa with polyclonal antibodies specific for (A and B) ATP synthase beta subunit (mitochondrial and chloroplast forms), (C) oxygen-evolving enhancer protein 1, and (D) germin-like protein (GLP). Total foliar proteins were extracted from wheat leaves at 12, 24 or 36 h after infiltration with 1 μM Ptr ToxB in 20 mM sodium acetate buffer, pH 4.6 ('Ptr ToxB' lane) or buffer alone ('Control' lane), and subjected to protein gel blot analysis with the respective antibodies. The upper band (1) in panel D corresponds to spot 43 in Fig. 2-2, whilst the lower band (2) corresponds to spots 40 and 42.

and buffer-treated tissues only at 36 h, consistent with the decreased abundance of spots 40, 42 and 43 detected at 36 h by 2-DE (Table 2-1). At this time, the intensities of the upper and lower bands in the Western blot were decreased by 29.6 ± 3.7 % and 36.6 ± 4.8 %, respectively, in the toxin-treated vs. control treatments. In contrast, no significant differences in band intensities were observed between treatments when these were examined at 12 h and 24 h (data not shown).

2.4. Discussion

2.4.1. Inhibition of photosynthesis by Ptr ToxB

The current study provides the first direct evidence that Ptr ToxB inhibits photosynthesis in toxin-sensitive wheat. Application of the toxin led to significant declines in net photosynthetic rates within 12 h of treatment (Fig. 2-1), and preceded development of any visible chlorosis symptoms by at least 36 h. Previous studies have also shown that Ptr ToxB-induced declines in chlorophyll and carotenoid pigments are not detectable until 48 h after toxin treatment (Kim and Strelkov, 2007; Strelkov *et al.*, 1998). Therefore, the early decline in photosynthesis suggests, as we previously hypothesized, that inhibition of this process by Ptr ToxB results in the development of chlorosis, likely through the formation of reactive oxygen species (ROS) (Strelkov *et al.*, 1998).

2.4.2. Proteome level changes induced by Ptr ToxB

To support our hypothesis that Ptr ToxB causes a disruption in photosynthetic processes, as well as to evaluate other alterations in host metabolism caused by the toxin, we undertook a proteomic analysis of Ptr ToxB-

induced changes in wheat leaves at 12, 24 and 36 h after treatment (Fig. 2-2). These times were chosen because they preceded the appearance of visible symptoms, and could thus help to understand the mechanisms of chlorosis development in toxin-sensitive wheat. The proteins identified in this analysis are discussed below, both within the context of toxin action and with respect to reported functions in the host and in other pathosystems.

2.4.3. Energy metabolism

A total of 17 proteins with roles in energy metabolism were found to be differentially abundant after Ptr ToxB treatment (Fig. 2-3), 16 of which have been implicated in photosynthesis-related processes. OEE1, which was up-regulated at 24 h (Table 2-1, Fig. 2-5), is part of the oxygen evolving complex (OEC) of photosystem II (PSII) and is essential for PSII stability (Mayfield *et al.*, 1987). Deletion mutants for OEE1 were found to be more sensitive to photoinhibitory treatments (Philbrick *et al.*, 1991). The increased abundance of this protein in toxin-treated leaves may thus reflect an attempt by the host to maintain normal function in response to possible disruptions in the photosynthetic electron transport chain (PETC). Indeed, the decreased abundance, at 12 h, of FNR, a thylakoid membrane-associated enzyme that catalyzes the transfer of electrons from reduced ferredoxin to NADP in the last step of the PETC (Carrillo and Vallejos, 1982), is suggestive of disruptions to photosynthetic electron transport shortly after toxin treatment. Significant declines were also observed in the abundance of several ATP synthase subunits, including a chloroplast precursor, at 12 h after treatment with Ptr ToxB (Table 2-1, Fig. 2-5). These declines could

indicate a disruption in the integrity of the chloroplasts or associated membranes, since ATP synthase is found in the thylakoid membranes, where it generates ATP in photophosphorylation. The abundance of a spot matching a mitochondrial ATP synthase beta subunit, however, was significantly increased at 24 h, suggesting more widespread disruptions to energy metabolism at the later time-points.

In addition to the identification of proteins with roles in the light reactions of photosynthesis, proteins with functions in the Calvin cycle were also found to be differentially abundant after treatment with Ptr ToxB (Table 2-1). RuBisCo activase was up-regulated in toxin-treated tissues at 24 h after infiltration, but was less abundant by 36 h. The initial increase in this enzyme may indicate an attempt by the host to maintain a normal photosynthetic metabolism, since RuBisCo activase is a nuclear-encoded chloroplast enzyme that activates RuBisCo, one of the key enzymes in photosynthetic carbon assimilation (Portis, 2003). Ultimately, however, levels of RuBisCo activase declined, likely as Ptr ToxB-induced damage became more widespread. Although no significant differences were detected with respect to the abundance of RuBisCo itself, it was difficult to distinguish some of the individual subunits on the 2-D gels, making quantification difficult. The abundance of TIM, which catalyzes the reversible interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (GAP), was also significantly altered, with an increase observed at 24 h after toxin-treatment. In plants, TIM occurs as two isoforms, namely a Calvin cycle enzyme found in the chloroplasts and a glycolytic enzyme found in the cytosol (Gottlieb, 1982; Mo *et al.*, 1973). The fact that one of the TIM spots corresponded to the chloroplast

precursor (Table 2-1) suggests that the Calvin cycle isoform was affected. The increased abundance of TIM at 24 h is consistent with the increased abundance of RuBisCo activase at the same time. Quantities of FBA, a glycolytic enzyme that also functions in the Calvin cycle, catalyzing the condensation of fructose-1,6-bisphosphate from dihydroxyacetone phosphate and GAP, were also significantly altered, as was the abundance of PRKase, a Calvin cycle enzyme catalyzing the biosynthesis of ribulose-1,5-bisphosphate. Changes in the levels of these enzymes further indicate alterations in the carbon assimilation process of photosynthesis.

2.4.4. Metabolism

The intensities of four spots matching plastid GS, which catalyzes the biosynthesis of Gln from Glu and plays a key role in the assimilation of ammonia produced from photorespiration and nitrate reduction (Wallsgrove *et al.*, 1987), were also significantly altered after toxin treatment (Table 2-1). Fluctuations in the levels of this enzyme could reflect Ptr ToxB-induced changes in cellular nitrogen metabolism, including photorespiration, a side reaction of photosynthesis. Quantities of MDH, which catalyzes the reversible interconversion of oxaloacetate into malate (Goward and Nicholls, 1994), were increased at 24 h. The expression and activity of various isoforms of MDH have been found to change in response to a variety of stresses (Jorge *et al.*, 1997; Kalir and Poljakoff-Mayer, 1981; Kumar *et al.*, 2000), and the increase in MDH observed in toxin-treated tissues may reflect oxidative stress resulting from the disruption of photosynthesis. The abundance of AdoMet synthetase 1 increased significantly at both 24 and 36 h after toxin treatment (Table 2-1). AdoMet synthetase catalyzes

the biosynthesis of *S*-adenosyl-L-methionine (AdoMet), which is a precursor of ethylene (ET). In plants, ET modulates various stress responses, playing important roles in defense, disease resistance and signaling pathways (Broekaert *et al.*, 2006). The increased abundance of AdoMet synthetase 1, therefore, suggests an increase in ET production, perhaps reflecting an up-regulation of host defense mechanisms. ET biosynthesis and the degradation of lipid membranes are also closely linked (Paulin *et al.*, 1986), and Bartoli *et al.* (1996) found a close association between ET production and antioxidant capacity in cut carnations.

2.4.5. Cell rescue, defense, and virulence

The quantities of a number of proteins involved in cell rescue and host defense were found to be altered after treatment with Ptr ToxB (Fig. 2-3). An increased abundance of Harpin binding protein 1 was detected at 24 h (Table 2-1). Harpins represent a group of effector proteins produced by some plant pathogenic bacteria, which, if infiltrated into non-host tissues, induce a variety of defense responses (Galán and Collmer, 1999). The specific role of Harpin binding protein 1 in the current context, however, remains unclear. In contrast, the increased abundance of APX at 24 h (Table 2-1), and the increase in total POX activity at 12, 24 and 36 h (Fig. 2-4), suggests an attempt by the host at detoxification of ROS, especially hydrogen peroxide, which may have accumulated as a result of the inhibition of photosynthesis (Fig. 2-1). In an earlier study with Arabidopsis, Karpinski *et al.* (1999) found that redox changes in electron transport through quinone B (Q_B) and/or plastoquinone (PQ) induced expression of *APX2*, a gene encoding ascorbate peroxidase. They also showed that expression of this gene

could be induced by hydrogen peroxide, suggesting that this molecule is involved in the acclimation to photooxidative stress (Karpinski *et al.*, 1999). The current results are consistent with these findings, and may serve to further support a toxin mechanism of action that involves the inhibition of electron transport.

The increased abundance of HSP70 at both 24 and 36 h may also reflect inhibition of photosynthesis and ROS-mediated damage. It was previously found that overexpression of the *HSP70B* gene, which encodes chloroplast-targeted HSP70 in the unicellular green alga *Chlamydomonas reinhardtii*, resulted in enhanced protection against photoinhibition and more rapid repair of PSII reaction centers (Schroda *et al.*, 1999). Similarly, Yokthongwattana *et al.* (2001) reported that HSP70B appears to be involved in repair of photodamaged PSII reaction centers in the green alga *Dunaliella salina*. The increased levels of HSP70 observed in the current study, therefore, suggest an activation of mechanisms to repair photooxidative damage induced by Ptr ToxB.

The abundance of proteins identified as GLP 1 decreased significantly at 36 h after toxin-treatment (Table 2-1), a decline confirmed by Western blotting analysis (Fig. 2-5D). Germins and GLPs have functions in many processes related to plant development and defense (Bernier and Berna, 2001). Some GLPs have been found to possess oxalate oxidase (OXO) activity, and a germin-like OXO accumulated in barley leaves inoculated with *Blumeria graminis* (DC.) Golovin ex Speer f. sp. *hordei* Em. Marchal, suggesting a function in defense via the production of hydrogen peroxide (Zhang and Collinge, 1995). Other GLPs have been reported to have superoxide dismutase (SOD) activity, and a GLP with SOD

activity (GLP4) contributed to quantitative resistance against *B. graminis* in wheat and barley (Christensen *et al.*, 2004). GLPs with protease activity or with ADP glucose pyrophosphatase or phosphodiesterase activity have also been reported in wheat and barley, respectively (Rodríguez-López *et al.*, 2001; Segarra *et al.*, 2003). In the former case, it was found that a GLP in the wheat leaf apoplast controlled serine protease activity in plants inoculated with *Septoria tritici* Berk. & M.A. Curtis (Segarra *et al.*, 2003). The defense-related roles described for GLP suggest that a Ptr ToxB-mediated decline in this protein could increase susceptibility to infection by *P. tritici-repentis*.

2.4.6. Protein fate

FtsH-like protein plastid fusion and/or translocation factor (Pftf) is a thylakoid membrane-bound, ATP-dependent metalloprotease belonging to the AAA protein superfamily, and is involved in PSII repair (Bailey *et al.*, 2002). The decline of this protein at 12 h after toxin treatment suggests damage to the thylakoid membranes, likely as a result of the formation of ROS, and is consistent with the declines in other PETC/thylakoid membrane-associated enzymes observed at this time.

2.4.7. Biogenesis of cellular components

The actin cytoskeleton can be an important component of plant defense mechanisms against fungal penetration and infection (Kobayashi and Hakuno, 2003; Kobayashi *et al.*, 1992), while RGP is involved in synthesis of cell wall hemicellulosic polysaccharides (De Pino *et al.*, 2007; Dhugga *et al.*, 1997). The increased abundance of actin and RGP in the current study, therefore, suggests an

attempt by the host to reinforce the cell walls in order to maintain the integrity of the cells.

2.4.8. Other differentially abundant proteins

The significance of the catalytic/coenzyme binding protein identified in this study is not clear, nor are the roles of five differentially induced spots corresponding to hypothetical or unclassified proteins. Interestingly, Ptr ToxB was not identified in the foliar protein extracts, probably because of its relatively small size and/or the low concentrations infiltrated into the host tissue. In a previous study, Ptr ToxB could not be detected in the secretome of *P. tritici-repentis* by 2-DE, although its production was confirmed by Western blotting (Cao *et al.*, 2009).

2.4.9. A model for Ptr ToxB action

In the current study, we demonstrated that treatment with Ptr ToxB results in a decline in photosynthesis (Fig. 2-1) that precedes the development of chlorosis or any detectable decline in chlorophyll (Kim and Strelkov, 2007; Strelkov *et al.*, 1998). Moreover, we found the toxin to have a profound effect on the abundance of foliar proteins, particularly those related to photosynthesis or associated with oxidative stress (Fig. 2-3). It should be noted that the total foliar proteome, rather than the chloroplast proteome, was examined. This dilution effect, therefore, serves to highlight the impact of Ptr ToxB on chloroplast-associated proteins, since these still constituted the largest group of differentially abundant spots (Table 2-1).

Based on these results and those of an earlier study (Strelkov *et al.*, 1998), we can begin to develop a hypothetical model for Ptr ToxB action in toxin-sensitive wheat. Within 12 h after infiltration, the toxin begins to significantly inhibit photosynthesis, although the mechanism for this inhibition is unknown. Nevertheless, by this time, the abundance of enzymes involved in the PETC, specifically FNR, is reduced, as is that of several Calvin cycle enzymes, PRKase and FBA. Also at 12 h, the levels of other thylakoid membrane-associated enzymes, including ATP synthase and FtsH-like protein Pftf, decline as well. Collectively, these results suggest a rapid disruption of photosynthetic mechanisms by Ptr ToxB, leading to the generation of ROS and, consequently, oxidative stress. Indeed, we previously found that p-benzoquinone, which reduces the half-life of the energetic triplet state of chlorophyll and also scavenges singlet oxygen (Fujimori and Livingston, 1957), prevented development of chlorosis (Strelkov *et al.*, 1998). In this study, the production of ROS is supported by the increase, at 24 h, of a number of proteins involved in PSII repair, detoxification of ROS, and the attenuation of oxidative stress. These include OEE1, APX, and HSP70. Total POX activity also increased significantly at 12, 24 and 36 h after toxin-treatment (Fig. 2-4). The increases in these proteins suggest that many of the normal photoprotective mechanisms of the host are still active, at least at this stage. There also appear to be other attempts by the host cells to maintain normal functions. For instance, at 24h, the Calvin cycle enzymes Rubisco activase and TIM are more abundant, as is FBA at 36 h.

In addition, the levels of actin and reversibly glycosylated polypeptide are increased after toxin treatment, perhaps to strengthen the host cell walls. The increase in AdoMet synthetase also suggests an up-regulation of host defense mechanisms, through increased biosynthesis of ET. As noted above, a close relationship between ET production and antioxidant capacity has been reported (Bartoli *et al.*, 1996), so this increase could also reflect a response to oxidative stress. In contrast to the up-regulation of these enzymes, the abundance of GLP decreases at 36 h, suggesting that certain components of the host defense response may be compromised by Ptr ToxB action, which would facilitate infection by toxin-producing isolates of *P. tritici-repentis*. Fluctuations in other proteins involved in cellular metabolism, such as plastid GS, likely reflect profound physiological disturbances and/or the localization of some of these proteins in the plastids or chloroplasts. Regardless of attempts by the host to control Ptr ToxB-induced damage, however, photosynthesis rates continue to decline (Fig. 2-1), and by 48 h the levels of chlorophyll also begin to decrease, likely as a consequence of photooxidation (Strelkov *et al.*, 1998). The inhibition of photosynthesis, and the resulting increase in ROS, may eventually overwhelm the photoprotective systems of the host, and damage may become widespread. Indeed, following the development of visible chlorosis at 48 to 72 h, the most severely affected tissue becomes necrotic, which would favor colonization by *P. tritici-repentis*.

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3. Transformation of a Ptr ToxB non-producing isolate of *Pyrenophora tritici-repentis* with the *ToxB* gene confers pathogenicity on a toxin-sensitive wheat genotype

3.1. Introduction

Pyrenophora tritici-repentis (Died.) Drechsler [anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker] is a necrotrophic ascomycete pathogen that causes tan spot of wheat, a destructive foliar disease of worldwide importance (De Wolf *et al.*, 1998; Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). Tan spot can cause yield losses of up to 50% under conditions favorable for the pathogen (Rees *et al.*, 1982; Shabeer and Bockus, 1988), and is distinguished by the development of two discrete symptoms, tan necrosis and extensive chlorosis, on infected leaves. The development of these symptoms results from the differential production of at least three host-specific toxins (HSTs), known as Ptr ToxA, Ptr ToxB and Ptr ToxC, by isolates of *P. tritici-repentis* (Lamari and Strelkov, 2010). HSTs are a class of effectors produced by necrotrophic plant pathogens, which result in increased fungal virulence and toxicity only on host genotypes that express matching sensitivity genes (Friesen *et al.*, 2008). The first HST identified from *P. tritici-repentis*, Ptr ToxA, is a 13.2 kDa proteinaceous necrosis-inducing toxin that is encoded by a single copy gene, *ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). The second HST, Ptr ToxB, is a protein 6.6 kDa in size that is encoded by the *ToxB* gene and induces chlorosis on toxin-sensitive wheat genotypes (Martinez *et al.*, 2001; Strelkov and Lamari, 2003; Strelkov *et al.*, 1999). The third HST 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 does not appear to be a protein, but instead has been described as a polar, non-ionic compound of low molecular mass (Effertz *et al.*, 2002).

Isolates of *P. tritici-repentis* are classified into races based on their ability to produce the Ptr toxins and thereby specifically cause necrosis or chlorosis on members of a host differential set. To date, eight races of *P. tritici-repentis* have been identified on three effective wheat differential genotypes, consisting of the cv. Glenlea and lines 6B365 and 6B662 (Lamari *et al.*, 1995; Lamari *et al.*, 2003; Strelkov *et al.*, 2002). Race 1 isolates of the fungus produce Ptr ToxA and Ptr ToxC, while race 2 isolates produce only Ptr ToxA. Isolates classified into races 3 and 5 produce Ptr ToxC or Ptr ToxB, respectively (Strelkov and Lamari, 2003), while those classified as race 6 produce both Ptr ToxC and Ptr ToxB (Strelkov *et al.*, 2002). Race 7 isolates also produce two HSTs, Ptr ToxA and Ptr ToxB, while race 8 isolates produce all three known toxins (Lamari *et al.*, 2003; Strelkov and Lamari, 2003). Race 4 isolates do not produce active forms of any of the HSTs and are non-pathogenic (Strelkov and Lamari, 2003). While there have been preliminary reports of additional races of *P. tritici-repentis*, these await full characterization (Manning *et al.*, 2002; Meinhardt *et al.*, 2003).

Ptr ToxB has been shown to inhibit photosynthesis in sensitive wheat genotypes, resulting in chlorophyll photooxidation and the development of chlorosis (Kim *et al.*, 2010; Strelkov *et al.*, 1998), although its mode of action is not completely understood. The *ToxB* gene consists of a 261-bp open reading

frame (ORF) that encodes a 23 amino acid (aa) signal peptide and a 64 aa mature protein (Martinez *et al.*, 2001; Strelkov and Lamari, 2003). Unlike *ToxA*, *ToxB* occurs as a multiple copy gene in isolates of *P. tritici-repentis* that possess Ptr ToxB activity (Lamari *et al.*, 2003; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). For instance, 8-10 copies of *ToxB* have been found in strongly pathogenic 'wild-type' race 5 isolates of the fungus, while only 2 copies of the gene were reported in a weakly pathogenic isolate of the same race (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Homologs of *ToxB* are also found as single copies in isolates belonging to races 3 and 4 of *P. tritici-repentis* (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006). Although these races do not possess any known Ptr ToxB activity, the respective *ToxB* homologs have been found to be expressed at low levels in conidia of a race 3 isolate (Strelkov *et al.*, 2006) and in conidia and mycelia of a race 4 isolate (Amaike *et al.*, 2008). Recently, homologs of *ToxB* have also been reported in other ascomycete fungi, including *Pyrenophora bromi* (Died.) Drechsler (Andrie *et al.*, 2008). While the form of Ptr ToxB in race 4 isolates of *P. tritici-repentis* appears to have little or no chlorosis-inducing activity on toxin-sensitive wheat (Figueroa Betts *et al.*, 2011; Kim and Strelkov, 2007), Pb ToxB, the form of the toxin from *P. bromi*, does (Andrie and Ciuffetti, 2011).

Silencing of the *ToxB* gene in a wild-type race 5 isolate of *P. tritici-repentis* revealed that Ptr ToxB is not only a pathogenicity factor essential for tan spot development, but that it also serves as a virulence factor contributing quantitatively to disease severity (Aboukhaddour *et al.*, 2012). In an effort to better understand this dual nature of Ptr ToxB as both a pathogenicity and

virulence factor, a biologically active 'wild-type' copy of the *ToxB* gene was transformed from a race 5 isolate of *P. tritici-repentis* into a Ptr ToxB non-producing race 2 isolate of the fungus, which does not possess *ToxB* or any of its homologs. The ability of the transformants to cause disease and the degree of this pathogenicity on Ptr ToxB-sensitive and insensitive wheat genotypes was then assessed, with the hypothesis that toxin production would permit disease development on the Ptr ToxB-sensitive wheat genotype to an extent correlated with the amount of Ptr ToxB produced.

3.2. Materials and methods

3.2.1. Fungal isolates and growth conditions

Isolates 86-124 and Alg3-24, representing races 2 and 5 of *P. tritici-repentis*, respectively (Lamari and Bernier, 1989b; Lamari *et al.*, 1995), were used throughout this study. The race 2 isolate was collected in Manitoba, Canada (Lamari and Bernier, 1989b) and produces Ptr ToxA, encoded by the single copy gene *ToxA*, but does not possess a copy of the *ToxB* gene or any of its homologs. The race 5 isolate Alg3-24, which was collected in eastern Algeria, possesses 8-10 copies of *ToxB*, and produces active Ptr ToxB (Strelkov *et al.*, 2006).

To produce fungal mycelium, the isolates and transformed strains of *P. tritici-repentis* were grown at room temperature under darkness in 9-cm diameter Petri dishes filled with V8-potato dextrose agar (PDA) (Lamari and Bernier, 1989a) or in 300-mL Erlenmeyer flasks filled with liquid Fries' medium (Dhingra and Sinclair, 1985) amended with 0.1% yeast extract. Fungal conidia were produced on V8-PDA as previously described (Lamari and Bernier, 1989a).

Unless otherwise stated, all transformants were selected, grown and maintained on V8-PDA or Fries' medium to which 200 µg/mL or 150 µg/mL hygromycin B (Invitrogen, Burlington, ON, Canada), respectively, had been added.

3.2.2. Amplification of the *ToxB* gene

To amplify the *ToxB* ORF from race 5 isolate Alg3-24, a lyophilized mycelial mat was prepared and total genomic DNA was extracted as previously described (Kim and Strelkov, 2007). The forward primer ToxB6-*Xho*I (5'-CCACTCGAGTACAGTAATCTCTTCTACGCT-3') and reverse primer ToxB4-*Hin*dIII (5'-CGAAAGCTTCCCTATACCTAATGTAGGG-3') were used to amplify a 432 bp fragment containing the entire 261 bp region of the ORF, which included the region coding for the signal peptide of Ptr ToxB from the isolate Alg3-24. An *Xho*I or *Hind*III restriction site (underlined) was included in the forward and reverse primers, respectively, to enable insertion of the amplicon into a fungal transformation vector. A standard PCR was conducted in a 25 µL reaction volume consisting of 1 × PCR buffer, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.2 µM each primer, 100 ng DNA template and 1.0 unit of Platinum® *Taq* DNA polymerase (Invitrogen). Reaction conditions consisted of an initial heat denaturation step at 95°C for 5 min, followed by 30 cycles of 94°C for 50 s, 55°C for 50 s, and 72°C for 50 s, and a final extension at 72°C for 2 min. All PCR amplifications were conducted in a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA).

The amplification products were resolved on 1% (w/v) agarose gels and the corresponding bands were excised from the gels under ultraviolet light, with the amplicons extracted from the agarose using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON, Canada). The PCR products were sequenced at a commercial facility (Macrogen, Rockville, MD, USA) using BigDye Terminator cycling conditions on a 3730xl DNA Analyzer (Applied Biosystems), and once the correct sequence was confirmed, the purified amplicons were digested with *Xho*I and *Hin*dIII (New England Biolabs, Pickering, ON, Canada) for 2 hours at 37°C as per the manufacturer's instructions.

3.2.3. Construction of the fungal transformation vector

To construct a fungal transformation vector, the pSilent1 plasmid (Nakayashiki *et al.*, 2005), containing the *hygromycin B phosphotransferase* (*hph*) and ampicillin resistance genes as selection markers, was obtained from the Fungal Genetics Stock Center (McCluskey *et al.*, 2010). To clone the *ToxB* insert, purified pSilent1 plasmid was digested with *Xho*I and *Hin*dIII (New England Biolabs) for 4 hours at 37°C as per the manufacturer's instructions. The restriction enzyme-digested *ToxB* amplicon obtained above was then ligated into the 5' multiple cloning site of the pSilent1 plasmid with a Rapid DNA Ligation Kit (Roche, Laval, QC, Canada) as per the supplier's protocol, resulting in a transformation vector designated as pSilent-ToxB1. The ligation mix was then used to transform chemically competent *Escherichia coli* DH5α cells (Invitrogen) as per the manufacturer's instructions, which were plated on Luria-Bertani [LB (Miller)] agar medium containing ampicillin (100 µg/mL) and incubated

overnight at 37°C. Colony PCR (Güssow and Clackson, 1989) was conducted with randomly selected bacterial colonies using the *ToxB*-specific primers ToxB6-*Xho*I/ToxB4-*Hin*dIII, under the same conditions as described earlier, to verify the presence of the pSilent-ToxB1 construct. Colonies were sub-cultured in liquid LB medium containing ampicillin (100 µg/mL) and the pSilent-ToxB1 fungal transformation vector was purified using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced as above to ensure that the correct *ToxB* insertion was present in the construct.

3.2.4. Fungal protoplast preparation

Fungal protoplasts were prepared as per the methods of Ciuffetti *et al*. (1997) and Aboukhaddour *et al*. (2009) with some minor modifications. Briefly, conidia of the race 2 isolate 86-124 were harvested in sterile 0.25 × potato-dextrose broth (PDB) medium and adjusted to a final concentration of 5×10^5 conidia/mL. A 500 mL aliquot of this conidial suspension was incubated in darkness at room temperature for an 18 hour period with agitation (100 r/min). The germinating conidia and hyphal fragments were filtered through sterile mira-cloth and washed with sterile-distilled water. After removing excess water, 3 g (fresh weight) of the conidial suspension was placed in a sterile Petri dish (5-cm-diameter) and incubated in 8 mL of filter-sterilized cell-wall degrading enzyme mix [1.2 M $MgSO_4$, 0.15 M sorbitol (Sigma-Aldrich, St. Louis, MO), 20 mM potassium phosphate osmotic buffer, pH 5.8, containing 40 mg of driselase (Sigma-Aldrich), 160 mg of lysing enzymes (Sigma-Aldrich), 20 mg of β-glucuronidase Type H-1 (Sigma-Aldrich), and 50 mg of yatalase (Takara Mirus

Bio, Madison, WI, USA)] for 3 h at 33°C in darkness with gentle agitation (50 r/min). The resulting protoplast suspension was then filtered through a sterile nylon mesh and the remaining conidia and hyphal fragments were rinsed twice with filter-sterilized STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$) to collect more protoplasts, resulting in a final volume of 40 ml protoplast suspension. The protoplasts were collected by centrifugation at 3000 × g for 10 min at 4°C, washed once in STC, and re-suspended in 100 µl of filter-sterilized STC buffer. The concentration of protoplasts was measured with a hemocytometer and adjusted to 1×10^7 protoplasts/mL in sterile STC, polyethylene glycol (PEG) solution [40% (w/v) PEG 4000, 50 mM Tris, pH 7.5, 50 mM $CaCl_2$] and dimethyl sulfoxide (DMSO, Sigma) buffer (80:20:1, v/v/v). The final suspension was stored as 100 µl aliquots at -80°C until needed.

3.2.5. Transformation of *P. tritici-repentis*

Fungal transformation was performed following a procedure modified from Ciuffetti *et al.* (1997). Briefly, 100 µl of the protoplast suspension (1×10^7 protoplasts/mL) prepared above was mixed gently with 1 µl of 0.1 M spermidine (Fluka, Oakville, ON, Canada) and 3 µg of the pSilent-ToxB1 vector, and then incubated on ice for 60 min. One mL of 40% PEG 4000 solution was then added to this suspension, gently mixed and incubated for 20 min at room temperature. The resulting protoplast-vector-PEG suspension was mixed with 4 mL of molten regeneration medium (RM), containing 1.2 M sorbitol, 0.1% yeast extract, 0.1% casein hydrolysate (Fluka), and 0.8% agar. A 5 mL aliquot of this mixture was spread evenly on top of 15 ml of solidified RM (containing 1.5% agar) in a 9-cm-

diameter Petri dish, and incubated at room temperature for 24 h in darkness. Following this incubation, the protoplasts were covered with 5 mL of molten RM containing 1.5% agar and 3.75 mg/mL hygromycin B (Invitrogen), resulting in a final concentration of 150 µg/mL hygromycin B. After an additional incubation at room temperature in darkness for 7 days, the hyphal tips of the emerging fungal colonies were transferred individually to 9-cm-diameter Petri dishes filled with V8-PDA amended with 200 µg/mL hygromycin B. Each emerging transformant was sub-cultured by hyphal tip transfer onto V8-PDA amended with 200 µg/mL hygromycin B at least three times.

3.2.6. Fungal growth rates

The growth rate of selected transformants and the non-transformed isolate 86-124 was monitored by measuring the colony diameter on V8-PDA in two directions at right angles to each other over a period of 6 days, with the cultures maintained in darkness at room temperature. Each strain was cultured in triplicate (i.e., three replications or Petri dishes per strain), with an average colony diameter calculated for each treatment at 6 days. The experiment was repeated three times.

3.2.7. PCR analysis

To determine whether or not there had been successful ectopic integration of the *hph* and *ToxB* genes into the genome of the transformed strains of *P. tritici-repentis*, total genomic DNA was extracted from each transformant and from the non-transformed isolates 86-124 and Alg3-24, as described above. For amplification of the *hph* gene, a set of *hph*-specific primers consisting of forward primer HPHF (5'-GAATTCAGCGAGAGCCTGAC-3') and reverse primer

HPHR (5'-GATGTTGGCGACCTCGTATT-3') was used in a PCR analysis of the genomic DNA, with a 453-bp amplicon expected. For amplification of the *ToxB* gene, the *ToxB*-specific primers consisting of forward primer ToxB6-*Xho*I and reverse primer ToxB4-*Hin*dIII were used. The PCR conditions were identical to those described above.

3.2.8. Southern blotting

For Southern blot analysis, genomic DNA was extracted from 40 mg of lyophilized mycelium as above, followed by digestion with the restriction endonuclease *Bam*H1, which does not cut within the *ToxB* ORF. Briefly, 15 μg of genomic DNA from each sample was resolved by agarose (1%) gel electrophoresis and blotted onto a Hybond N^+ nylon membrane (Amersham Biosciences, Oakville, ON, Canada) with 20× saline-sodium citrate (SSC) buffer (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0). The DNA was cross-linked by UV irradiation (245 nm). DNA labeling, hybridization and immunological detection were conducted using a DIG DNA Labeling and Detection Kit (Roche) according to the manufacturer's recommendations. Membranes were blotted with a 245-bp *ToxB* cDNA probe amplified with the primers ToxB1 (5'-GACTACCATGCTACTTGCTGTG-3') and ToxB2 (5'-AACAACGTCCTCCACTTTGC-3') as described above; the probe was labeled with digoxigenin (DIG) through a random primed labeling method (Roche). Following membrane washing, blocking and incubation with an Anti-DIG-AP antibody solution, the hybridizing bands were visualized by addition of a color substrate solution containing NBT/BCIP as per the manufacturer's instructions

(Roche). Blot images were recorded with a GS-800 Calibrated Densitometer (Bio-Rad).

3.2.9. Ptr ToxB production

Production of Ptr ToxB was assessed by Western blotting analysis of the total soluble protein from concentrated and dialyzed fungal culture filtrates. Briefly, culture filtrates from 21-day-old cultures of *P. tritici-repentis* isolates and transformants were harvested by vacuum filtration through Whatman No. 1 filter paper, followed by filtration through 0.45 µm cellulose nitrate membrane filters. The filtrate was flash-frozen at –80°C and lyophilized in a freeze-drier (Labconco Corp., Kansas City, MO, USA). The lyophilized residues were re-dissolved in 20 mM sodium acetate buffer (pH 4.6), dialyzed against water and lyophilized again. The total soluble protein in each sample was resolved by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Cao *et al.*, 2009). The membranes were incubated with primary polyclonal antibodies raised in rabbits against His-tagged Ptr ToxB (Cao *et al.*, 2009), followed by incubation with horseradish peroxidase-conjugated goat antirabbit IgG (Bio-Rad). Reacting bands were detected with a tetramethyl benzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Blot images were recorded with a GS-800 Calibrated Densitometer (Bio-Rad) and the Ptr ToxB band intensities were quantified using ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA); band intensities in the transformants are expressed relative to the intensity in the wild-type Ptr ToxB-producing isolate Alg3-24. Values represent the average intensity from two

biological replications, each of which included three technical replicates. Means (± standard error) are presented.

3.2.10. Bioassays

The pathogenicity of the transformed strains and wild-type isolates of *P. tritici-repentis* was assessed on two hexaploid wheat genotypes, line 6B662 and cv. Salamouni. Line 6B662 is sensitive to Ptr ToxB but insensitive to Ptr ToxA, while cv. Salamouni is insensitive to both toxins. Seeds were sown in 12.7-cm-diameter plastic pots filled with Sunshine LA4 Aggregate Plus Professional Growing Mix (Sun Gro Horticulture, Vancouver, BC, Canada) at a density of 4-5 seedlings per pot. The plants were maintained in a growth cabinet at 21/18°C (day/night) with a 16 hour photoperiod (light intensity of ≈180 µmol photons m^{-2} s^{-1}) and 60% relative humidity. Seedlings were inoculated at the 2-3 leaf stage with a conidial suspension (3000 conidia/mL) to which 10 drops of Tween 20 (polyoxyethylene sorbitan monolaurate) per liter had been added. The inoculum was applied to the leaves with an atomizer (Fisher Scientific, Ottawa, Canada). Following inoculation, the seedlings were placed in a humidity chamber (≥ 95% relative humidity) in darkness for 24 h, at which point they were transferred back to a growth cabinet and maintained as above. The plants were watered and fertilized as required. Six days after inoculation, the second and third leaves of the inoculated seedlings were collected and the total leaf and lesion areas were measured using Assess 2.0 Image Analysis Software (Lamari, 2008). The inoculation experiments were repeated three times, with three replicates (pots) per

treatment. Five randomly selected leaves from each replicate were analyzed, and the means (± standard error) are presented.

3.3. Results

3.3.1. Screening and selection of *ToxB*-transformed strains of *P. tritici-repentis*

After confirming that the wild-type *ToxB* gene from race 5 isolate Alg3-24 of *P. tritici-repentis* was correctly inserted into the pSilent-ToxB1 vector, protoplasts of race 2 isolate 86-124 were transformed with this vector via PEG-mediated fungal transformation, with the *hph* gene employed as a selection marker. Approximately 150 colonies were generated on hygromycin-containing RM, 33 of which were selected three times on V8-PDA-hygromycin medium by hyphal-tip transfer. No colonies lacking the *hph* gene were detected by PCR on the hygromycin-containing RM. A total of five transformants were selected for further analysis, and are denoted as Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27.

3.3.2. PCR analysis

PCR analysis of total genomic DNA from the transformed strains using *ToxB*- and *hph*-specific primers confirmed the presence of *ToxB* and *hph* bands of the expected size in Ptf1, Ptf2, Ptr3 and Ptf27 (Fig. 3-1). However, while a band corresponding to *hph* was also obtained from genomic DNA of Ptf20, this transformant lacked a *ToxB* band. As expected, neither the *ToxB* nor the *hph* bands could be amplified from DNA of the non-transformed race 2 isolate 86-124 (Fig. 3-1), while only the *ToxB* band could be amplified from the Ptr ToxB-producing race 5 isolate Alg3-24 (data not shown).

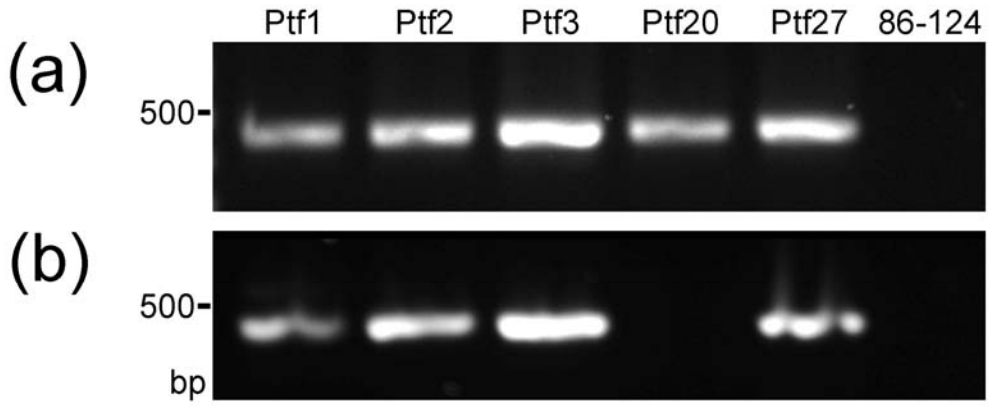


Figure 3-1. PCR amplification of the *hygromycin phosphotransferase* (*hph*) (A) and *ToxB* genes (B) from transformed strains and the wild-type race 2 isolate 86-124 of *Pyrenophora tritici-repentis*. (A) The *hph*-specific primers (HPHF and HPHR) amplified a 453-bp product from the five transformed strains Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27, but not from the wild-type isolate 86-124; (B) The *ToxB*-specific primers (ToxB6-*Xho*I and ToxB4-*Hin*dIII) amplified a 432-bp product from Ptf1, Ptf2, Ptf3 and Ptf27, but not from Ptf20 or 86-124. Negative controls in both PCR analyses did not produce any bands (not shown).

3.3.3. Southern blot analysis

Southern blot analysis with a DIG-labeled *ToxB* probe revealed the presence of multiple hybridizing bands in total genomic DNA of the transformants Ptf1, Ptf2, Ptf3 and Ptf27, and of the race 5 isolate Alg3-24 (Fig. 3-2). In contrast, no hybridizing *ToxB* band was observed in Southern blots of the race 2 isolate 86-124 or the transformant Ptf20. The presence of a band just below the 2.0 kb marker represents the 1963 bp transcriptional unit of *ToxB* from pSilent-ToxB1, consisting of a 1531 bp fragment from pSilent1 and a 432 bp fragment from the cloned *ToxB* gene following digestion with *Bam*H1.

3.3.4. Growth of transformants

No differences were observed with respect to the general colony morphology of the transformed strains, particularly when compared to the non-transformed isolate 86-124 (Fig. 3-3A). Similarly, no significant (p <0.05) differences were observed between 86-124 and any of the transformants with respect to growth rate, as measured by colony diameter after 6 days of incubation (Fig. 3-3B).

3.3.5. Western blot analysis

The amount of Ptr ToxB protein in the transformed strains and the wild-type isolates 86-124 and Alg-3-24 of *P. tritici-repentis* was evaluated by Western blotting analysis with polyclonal antibodies raised against His-tagged-Ptr ToxB (Cao *et al.*, 2009). The antibodies reacted with a single band of the expected size (~6-7 kDa) in concentrated culture filtrates from transformants Ptf1, Ptf2, Pft3

Figure 3-2. Southern blot analysis of genomic DNA from transformed strains and the wild-type race 2 isolate 86-124 of *Pyrenophora tritici-repentis* with a digoxigenin-labeled *ToxB* probe. Fifteen µg of *Bam*H1-digested genomic DNA of each strain was loaded per lane. M = DNA Molecular Weight Marker (MWM) II, Digoxigenin-labeled (200 ng; Roche); Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27 = strains of *P. tritici-repentis* transformed with the vector pSilent-ToxB1; 86-124 = the wild-type race 2 isolate; and Alg3-24 = the wild-type race 5 isolate of *P. tritici-repentis* (original source of the *ToxB* gene and included as a control). Note the presence of multiple hybridizing bands in Ptf1, Ptf2, Ptf3 and Ptf27, but absence of bands in Ptf20 and 86-124.

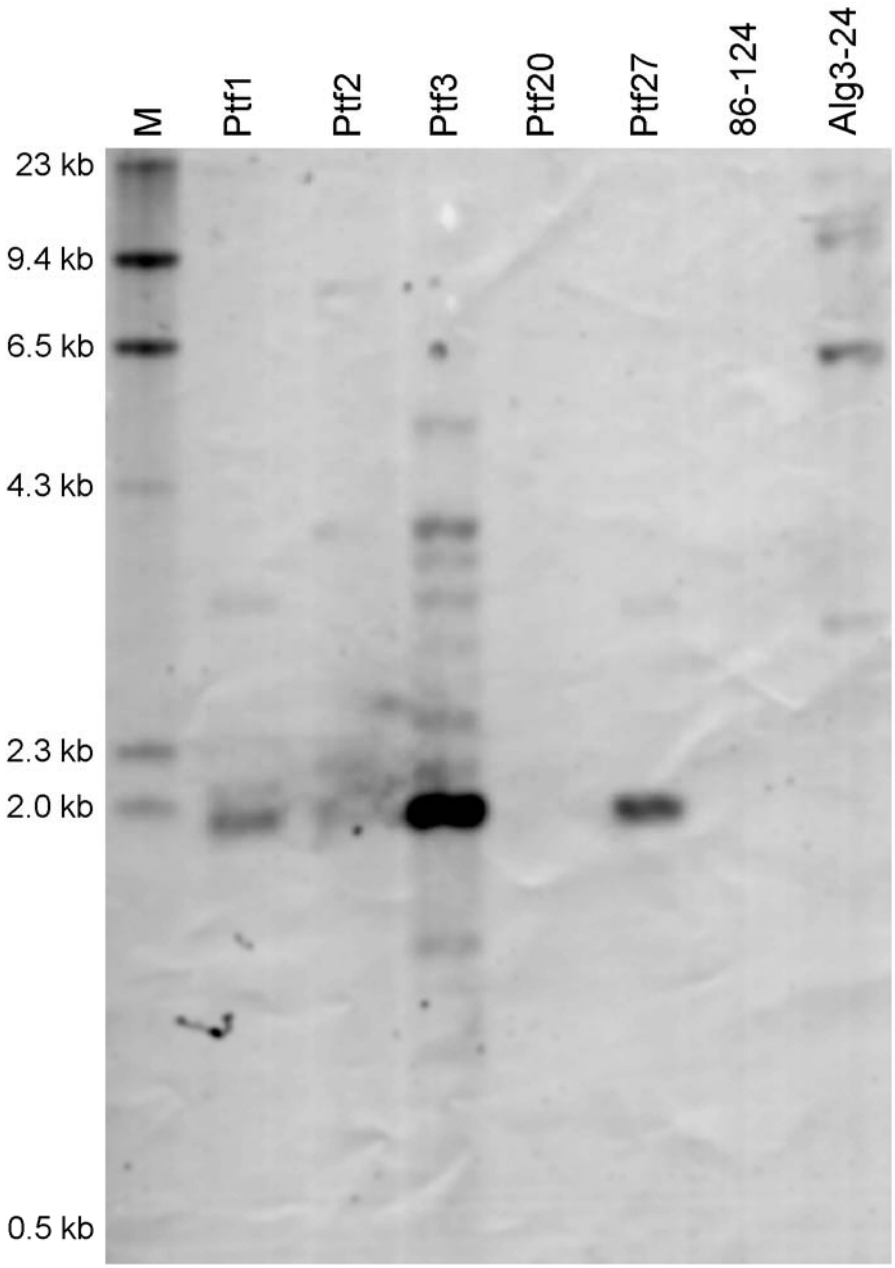
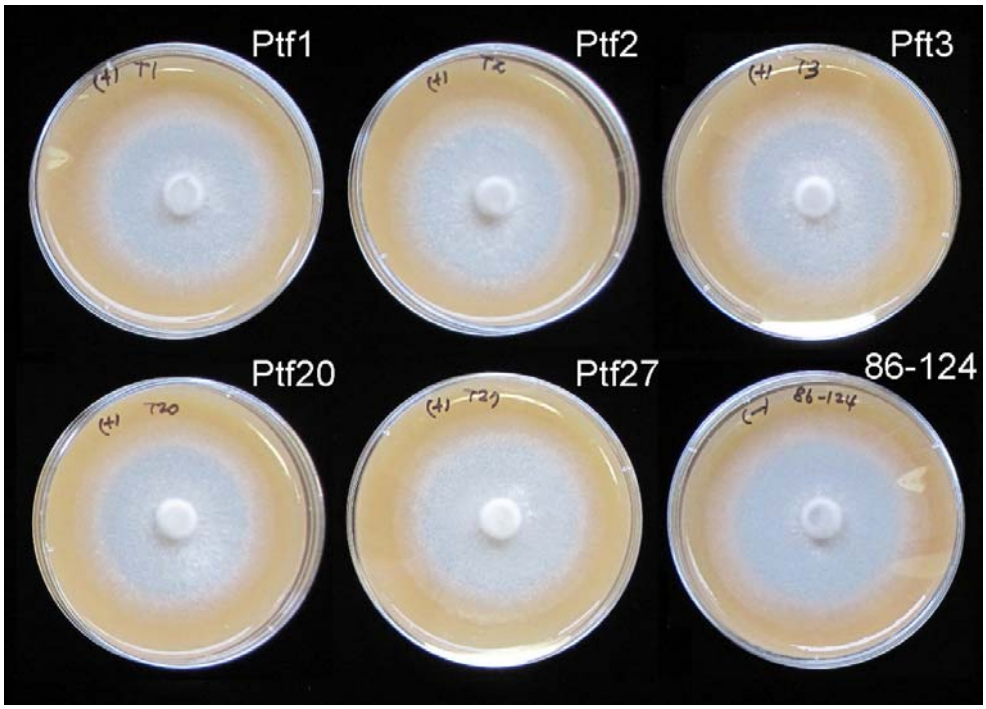
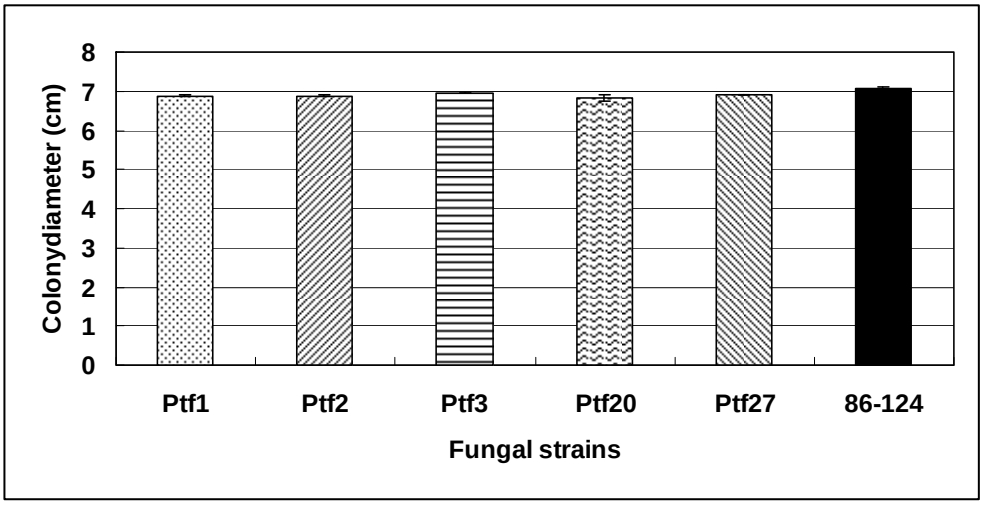

M
Ptf1
Ptf2
Ptf3
Ptf20
Ptf27
86-124
Alg3-24
23 kb
9.4 kb
6.5 kb
4.3 kb
2.3 kb
2.0 kb
0.5 kb

Figure 3-3. Growth of the *ToxB*-transformed hygromycin-resistant strains Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27 of *Pyrenophora tritici-repentis*. The wild-type isolate 86-124 of *P. tritici-repentis* is also included for comparison. (A) Morphology of colonies after 6 days of growth on V8-potato dextrose agar medium (V8-PDA). (B) Colony diameter after 6 days of growth on V8-PDA. Colony diameter was measured at two points perpendicular to each other. The mean of three replications (cultures) is shown, with bars representing the standard error of the mean.

A


Ptf1
Ptf2
Pft3
Ptf20
Ptf27
86-124

B


Colonydiameter (cm)
0
1
2
3
4
5
6
7
8
Ptf1
Ptf2
Ptf3
Ptf20
Ptf27
86-124
Fungal strains

and Pft 27, as well as in concentrated culture filtrates of the Ptr ToxB-producing race 5 isolate Alg3-24 (Fig. 3-4).

However, no corresponding Ptr ToxB band was observed in culture filtrates of 86-124 or of the transformant Ptf 20. The intensity of the Ptr ToxB band in the transformants producing this HST was lower than in Alg3-24; the relative band intensity in culture filtrates of Ptf1 was 35% ± 2% of that in Alg3-24, followed by an intensity of 34% ± 4% in filtrates of Ptf3. The intensity of the bands produced by Ptf27 and Ptf2 were only 16% ± 7% and 13% ± 2%, respectively, of that in Alg3-24.

3.3.6. Bioassays

The transformed strains and wild-type isolates 86-124 and Alg3-24 produced only small necrotic flecks 6 days after inoculation onto the Ptr ToxA- and Ptr ToxB-insensitive wheat cv. Salamouni, representing a typical resistant reaction. In contrast, Alg3-24 and the transformants Ptf1, Ptf2, Ptf3 and Ptf27 were all able to induce chlorosis on the Ptr ToxB-sensitive line 6B662, although to varying degrees (Fig. 3-5). Six days after inoculation with Alg3-24, 29.3% ± 6.2% of the total leaf area on line 6B662 had turned chlorotic, while 8.6 ± 1.4% and 8.1% ± 1.3 % of the leaf area was chlorotic following inoculation with Ptf3 and Ptf1, respectively. At 6 days, the transformants Ptf27 and Ptf2 caused the development of chlorosis on 7.4% ± 1.4% and 6.6% ± 0.8% of the total leaf area, respectively (Fig. 3-6). Inoculation with Ptf20, which did not produce Ptr ToxB (Fig. 3-4), did not cause any chlorosis symptoms on wheat line 6B662, instead causing necrotic flecks similar to those observed on cv. Salamouni (Fig. 3-5).

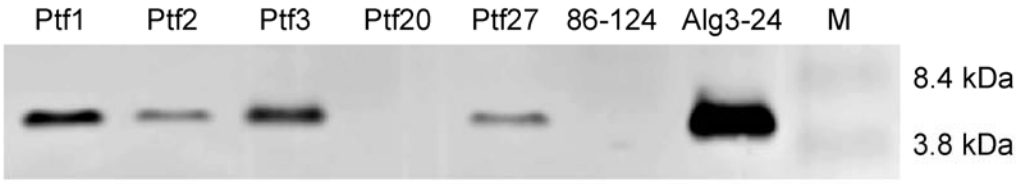
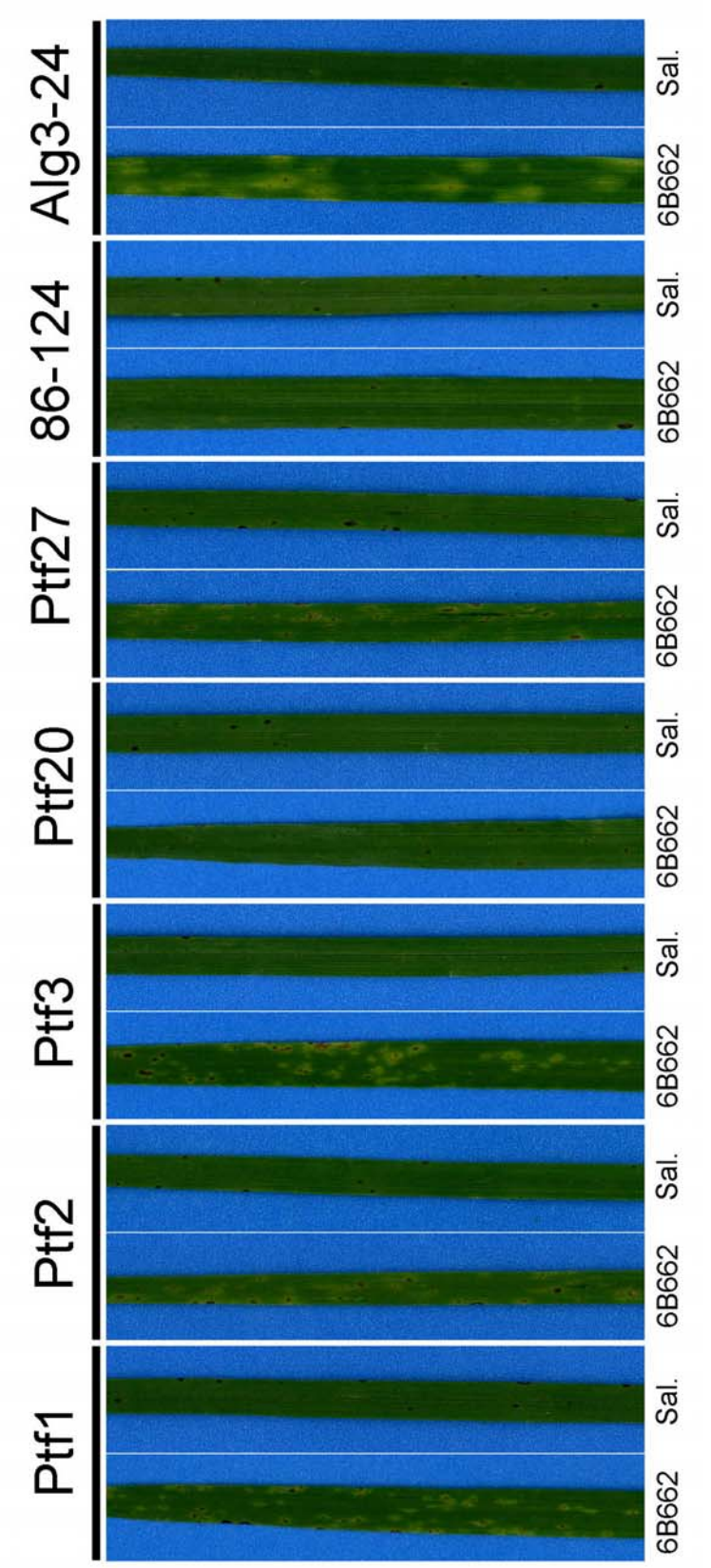


Figure 3-4. Western blot analysis of culture filtrates from transformed strains and the wild-type race 2 isolate 86-124 of *Pyrenophora tritici-repentis* with polyclonal antibodies specific to Ptr ToxB. Three µg of total protein from 21-day-old concentrated culture filtrates of each fungal strain were run in each lane. Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27 = strains of *P. tritici-repentis* transformed with the vector pSilent-ToxB1; 86-124 = the wild-type race 2 isolate; Alg3-24 = the wild-type race 5 isolate of *P. tritici-repentis* (original source of the *ToxB* gene encoding Ptr ToxB and included as a control); and M = Kaleidoscope Polypeptide Standards (10 µL; Bio-Rad). Note the presence of a band corresponding to Ptr ToxB in culture filtrates of Ptf1, Ptf2, Ptf3, Ptf27 and Alg3-24, but not in culture filtrates of Ptf20 or 86-124.

Figure 3-5. Symptoms induced by transformed strains and the wild-type race 2 isolate 86-124 and race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*. Leaves are shown 6-days after inoculation of the Ptr ToxB-sensitive wheat line 6B662 and the Ptr ToxB-insensitive cv. Salamouni. Ptf1, Ptf2, Ptf3, Ptf20 and Ptf27 = strains of *P. tritici-repentis* transformed with the vector pSilent-ToxB1; 86-124 = the wild-type race 2 isolate; Alg3-24 = the wild-type race 5 isolate of *P. tritici-repentis* (original source of the *ToxB* gene encoding Ptr ToxB).


Ptf1
Ptf2
Ptf3
Ptf20
Ptf27
86-124
Alg3-24
6B662
Sal.
6B662
Sal.
6B662
Sal.
6B662
Sal.
6B662
Sal.
6B662
Sal.
6B662
Sal.

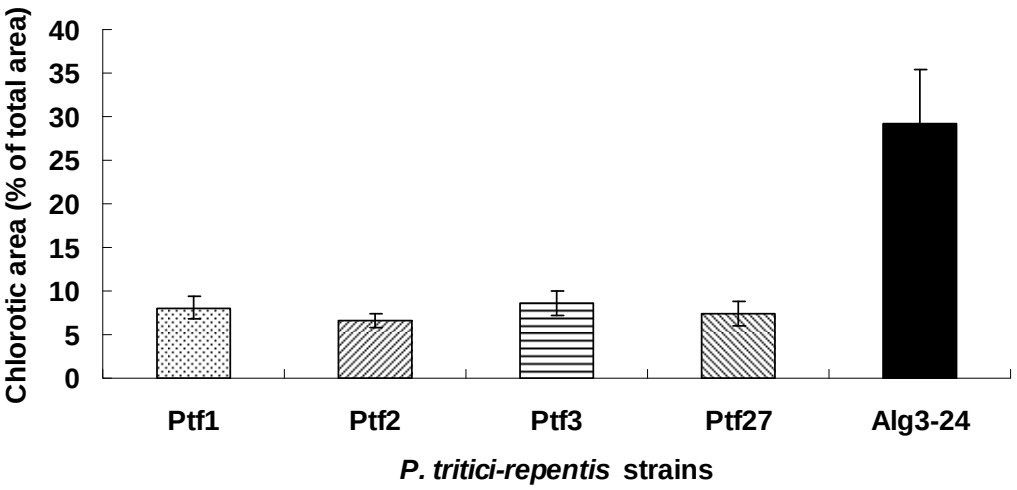


Figure 3-6. Percentage of total leaf area turning chlorotic in wheat line 6B662 in response to infection by transformed strains and race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*. Leaves were collected 6 days after inoculation and lesion areas were measured using Assess 2.0 Image Analysis Software (Lamari, 2008). Error bars indicate the standard error from three independent replicates per treatment. Ptf1, Ptf2, Ptf3 and Ptf27 = strains of *P. tritici-repentis* transformed with the vector pSilent-ToxB1; and Alg3-24 = race 5 isolate of *P. tritici-repentis* (original source of the *ToxB* gene encoding Ptr ToxB). The transformed strain Ptf20 and the wild-type race 2 isolate 86-124 did not produce Ptr ToxB and did not cause any chlorosis on line 6B662.

As expected, inoculation with the untransformed isolate 86-124 also failed to produce chlorosis on 6B662 and resulted only in necrotic flecking. The amount of chlorosis induced by the various strains and isolates on line 6B662 was generally well correlated with the amount of Ptr ToxB that they produced.

3.4. Discussion

In the toxin or inverse gene-for-gene model, a compatible interaction between a pathogen and host results when a HST produced by the pathogen is matched by a corresponding sensitivity gene in the host (Strelkov and Lamari, 2003). In the *P. tritici-repentis*-wheat pathosystem, Ptr ToxB sensitivity is conferred by the *Tsc2* gene, which is located on the short arm of chromosome 2B in tetraploid (*Triticum turgidum* L.) and hexaploid (*Triticum aestivum* L.) wheat (Abeysekara *et al.*, 2010; Friesen and Faris, 2004). Race 2 isolate 86-124 of *P. tritici-repentis* produces only the necrosis-inducing Ptr ToxA, and does not carry a *ToxB* homolog (Lamari *et al.*, 2003; Strelkov *et al.*, 2006). In this study, wild-type *ToxB* from race 5 isolate Alg3-24 was transformed into race 2 isolate 86-124, in order to assess whether the acquisition of this gene by itself could be a sufficient condition for pathogenicity on a Ptr ToxB-sensitive wheat genotype. As hypothesized, it was found that those transformants that acquired *ToxB* (Ptf1, Ptf2, Ptf3 and Ptf27; Fig. 3-1) could infect the Ptr ToxB-sensitive line 6B662, producing a range of chlorosis symptoms, while they remained non-pathogenic on the Ptr ToxA- and Ptr ToxB-insensitive wheat cv. Salamouni (Fig. 3-5). The transformant Ptf20, which carried the *hph* gene but not *ToxB*, and the non-transformed wild-type race 2 isolate 86-124, remained non-pathogenic on both

6B662 and ‘Salamouni’.

The ability of the *ToxB*-transformed strains of *P. tritici-repentis* to produce significant amounts of disease on the toxin-sensitive line 6B662 (Figs. 4 and 5) clearly demonstrates the role of Ptr ToxB as a pathogenicity factor for the fungus, *sensu* Yoder (1980). Moreover, since they retained their capacity to produce Ptr ToxA and infect the Ptr ToxA-sensitive differential cv. Glenlea (data not shown), the race designation of the transformants changed from the original race 2 classification of isolate 86-124, to a race 7 classification. Isolates classified as race 7 combine the virulence patterns of isolates belonging to races 2 (ToxA) and 5 (ToxB). This finding strongly supports the current race classification system for *P. tritici-repentis*, as defined by the ability of fungal isolates to produce certain HSTs or combinations of HSTs (Lamari *et al.*, 2003; Strelkov and Lamari, 2003).

In addition, and consistent with the hypothesis that Ptr ToxB serves both as a pathogenicity and virulence factor for *P. tritici-repentis* (Aboukhaddour *et al.*, 2012), the extent of the chlorosis induced by the fungal strains was correlated with the amount of Ptr ToxB they produced (Fig. 3-4). Thus, while the wild-type race 5 isolate Alg3-24 produced the greatest amount of toxin and caused the strongest chlorosis among all treatments, Ptf1 and Ptf3 produced the most Ptr ToxB and induced the strongest chlorosis among the transformed strains. The transformants Ptf27 and Ptf2, which produced lower amounts of Ptr ToxB, also caused weaker chlorosis, while the Ptr ToxB-non-producers 86-124 and Ptf20 did not cause any chlorosis on wheat line 6B662. A similar relationship was observed in naturally occurring isolates of *P. tritici-repentis*, with a race 5 isolate that

produced high amounts of Ptr ToxB causing significantly more chlorosis than a weak producer of the toxin, also classified as race 5 (Amaike *et al.*, 2008; Strelkov *et al.*, 2002). Likewise, silencing of the *ToxB* gene in the wild-type race 5 isolate Alg3-24 resulted in a number of silenced strains, each producing varying levels of Ptr ToxB, with the amount of toxin produced being well-correlated to tan spot severity on toxin-sensitive wheat (Aboukhaddour *et al.*, 2012). Transformation of a race 4 isolate of *P. tritici-repentis* also showed that the higher the *ToxB* copy number, the greater the virulence on a toxin-sensitive cultivar, although the amount of Ptr ToxB produced was not reported (Ciuffetti *et al.*, 2010). A similar situation has been reported recently in the wheat-*Stagonospora nodorum* (Berk.) Castell. & Germano pathosystem, in which higher levels of expression of the fungal effector SnToxA led to increased levels of Stagonospora nodorum blotch (Faris *et al.*, 2011).

The apparent dual nature of Ptr ToxB as both a fungal pathogenicity and virulence factor, along with the existence of forms of *ToxB* in other ascomycetes and in isolates of *P. tritici-repentis* that lack toxin activity, indicates that this protein could have a complex role(s) in the biology of necrotrophic fungal pathogens. It is worth noting, however, that that the acquisition of *ToxB* did not affect fungal growth rate or phenotype in the transformed strains, just as silencing of this gene in an earlier study did not affect growth rate or phenotype in the silenced strains (Aboukhaddour *et al.*, 2012). These findings are consistent with the suggestion that Ptr ToxB does not have any critical biological function in *P. tritici-repentis* aside from its role in pathogenicity (Strelkov and Lamari, 2003).

Nonetheless, while the exact function of *ToxB* and its homologs awaits further elucidation, it is clear that Ptr ToxB mediates compatibility between the tan spot fungus and different genotypes of its wheat host, as well as contributing to quantitative variation in the virulence of *P. tritici-repentis*.

3.5. References

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4. Expression of the host-selective toxin Ptr ToxB in the barley pathogen *Pyrenophora teres*

4.1. Introduction

The heterothallic ascomycete *Pyrenophora teres* Drechsler [anamorph: *Drechslera teres* (Sacc.) Shoemaker; syn. *Helminthosporium teres* Saccardo] is the causal agent of net blotch, an economically important foliar disease of cultivated barley (*Hordeum vulgare* L. emend. Bowden) worldwide (Mathre, 1997). *Pyrenophora teres* exists as two forms: *P. teres* f. *teres* and *P. teres* f. *maculata* (Smedegård-Petersen, 1971). While *P. teres* f. *teres* produces the characteristic net-like pattern of longitudinal brown necrotic streaks on infected barley leaves, *P. teres* f. *maculata* causes spot-type symptoms consisting of circular dark-brown lesions that are sometimes surrounded by chlorotic haloes (McLean *et al.*, 2009; Smedegård-Petersen, 1971). Yield losses can range from 10 to 40% in susceptible cultivars under conditions favorable for the pathogen (Mathre, 1997), with grain size and quality both reduced.

Three phytotoxins, toxin A [*N*-(2-amino-2-carboxyethyl) aspartic acid], toxin B (anhydroaspergillomarasmine A) and toxin C (aspergillomarasmine A), have been identified from culture filtrates of *P. teres*. These phytotoxins can cause necrosis and chlorosis on barley leaves, but do not reproduce the typical net- and spot-lesions associated with net blotch (Bach *et al.*, 1979; Smedegård-Petersen, 1977; Weiergang *et al.*, 2002). Toxins A and B do not determine pathogenicity in *P. teres*, but rather contribute to fungal virulence (Smedegård-Petersen, 1977). Other phytotoxic molecules, such as the pyrenolines A and B, have also been

isolated from culture filtrates of *P. teres* (Coval *et al.*, 1990). However, these isoquinoline derivatives showed no host specificity on barley or other plant species, and the symptoms they caused were not described in detail. More recently, a number of other chlorosis-inducing low molecular mass compounds from *P. teres* have been reported to be active in a temperature- and light-dependent manner, but they were also found to be host non-specific (Sarpeleh *et al.*, 2009).

In addition to the toxins A, B and C and other non-proteinaceous, low-molecular mass molecules, proteinaceous phytotoxic metabolites from culture filtrates of both *P. teres* f. *teres* and *P. teres* f. *maculata* have been identified. These molecules appear to be biologically active in a host-specific manner, producing brown-colored necrotic lesions or spots on a susceptible barley cultivar (Sarpeleh *et al.*, 2007). These proteinaceous metabolites are less than 100 kDa in mass and were reported to be heat-stable; their toxic activity was shown to be temperature and light-dependent, suggesting a role in the targeting of host organelles such as the chloroplast (Sarpeleh *et al.*, 2007, Sarpeleh *et al.*, 2008). However, the properties of these proteinaceous metabolites are not yet fully understood, and further study of the *P. teres*-barley interaction with regards to the involvement of host-specific toxin (HST) molecules is needed.

A close relative of *P. teres*, *Pyrenophora tritici-repentis* (Died.) Drechsler [anamorph *Drechslera tritici-repentis* (Died.) Shoemaker], the causal agent of tan spot of common and durum wheat (*Triticum aestivum* L. and *Triticum turgidum* L., respectively), produces multiple HSTs, known as Ptr toxins, that are responsible for the development of destructive foliar lesions on sensitive host genotypes

(Lamari and Strelkov, 2010; Strelkov and Lamari, 2003). These toxins serve as necrotrophic effectors for the fungus, and races of *P. tritici-repentis* are defined by their ability to produce the Ptr toxins in various combinations. In the wheat host, sensitivity to the Ptr toxins is conferred by independently inherited, dominant genes, which (presumably) specifically recognize the toxins, resulting in symptom development (Strelkov and Lamari, 2003).

Ptr ToxB, a 6.6 kDa proteinaceous HST produced by races 5, 6, 7 and 8 of *P. tritici-repentis* (Lamari *et al.*, 2003; Strelkov *et al.*, 1999; Strelkov *et al.*, 2002), causes extensive chlorosis on sensitive wheat genotypes via an inhibition of photosynthesis and the photooxidation of chlorophyll molecules (Kim *et al.*, 2010; Strelkov *et al.*, 1998). This toxin is encoded by a multiple copy gene, termed *ToxB*, which consists of a 261 bp open reading frame and encodes a 23 amino acid (aa) signal peptide and 64 aa mature toxin protein (Martinez *et al.*, 2001; Strelkov *et al.*, 2006). In addition to 'wild-type' *ToxB*, homologs of this gene have also been identified in Ptr ToxB non-producing isolates from races 3 and 4 of *P. tritici-repentis* (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006), as well as in other ascomycete fungi including *Pyrenophora bromi* (Died.) Drechsler [anamorph *Drechslera bromi* (Died.) Shoemaker] and in *P. teres* and *P. teres* f. *maculata* (Andrie *et al.*, 2008). The role of these *ToxB* homologs, however, is unknown. In order to gain insights into the function of *ToxB* in *P. teres*, and to determine whether the acquisition of Ptr ToxB producing ability is a sufficient condition for pathogenicity of this barley parasite on toxin-

sensitive wheat, we transformed a *P. teres* isolate with a biologically active 'wild-type' *ToxB* gene from *P. tritici-repentis*.

4.2. Materials and methods

4.2.1. Fungal material and growth conditions

The *P. teres* f. *maculata* isolate Dillon, originally collected from a hooded barley cultivar near Blackfalds, Alberta, was used in the fungal transformation studies as the recipient of the *ToxB* gene. The *P. tritici-repentis* race 5 isolate Alg3-24, originally collected from durum wheat in Algeria and a strong producer of Ptr ToxB (Lamari *et al.*, 1995; Strelkov *et al.*, 2002), was included as the source of the 'wild-type' *ToxB* gene and as a control in all inoculations.

To produce fungal mycelia, 'wild-type' and transformed strains of *P. teres* were grown on 20% (v/v) V8 agar (200 mL V8 juice, 3.0 g $CaCO_3$, 15 g agar per 1 L) or in liquid Fries' medium (Dhingra and Sinclair, 1985) amended with 0.1% yeast extract. Unless otherwise indicated, the V8 agar and Fries' medium used to culture transformed strains of *P. teres* contained 200 µg/mL or 150 µg/mL hygromycin B (Invitrogen, Burlington, ON, Canada), respectively. In order to produce conidia, a single-spore isolate of each transformed strain and the wild-type was incubated on 9 cm-diameter V8 agar plates at room temperature (RT) under continuous darkness for 5 to 7 days. After this period, 12 mm-diameter mycelial plugs were cut from each culture, placed on fresh V8 agar plates with the mycelium facing downwards, and incubated under the same conditions for another 5 to 6 days. To induce sporulation, new plugs were cut and transferred to fresh V8 agar plates, which were incubated for another 5 to 6 days at RT under a

12 h photoperiod with light provided by one near-UV and two cool white fluorescent lamps (Campbell *et al.*, 2003). Mycelium and conidia of *P. tritici-repentis* isolate Alg3-24 were produced as previously described (Lamari and Bernier, 1989).

4.2.2. Cloning of *ToxB*

To amplify the wild-type *ToxB* gene, genomic DNA was extracted from lyophilized mycelium of *P. tritici-repentis* race 5 isolate Alg3-24 using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol for plant tissue and with an additional three extractions in phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v). The forward primer ToxB6-*Xho*I (5'-CCACTCGAGTACAGTAATCTCTTCTACGCT-3'), containing an *Xho*I restriction site (underlined), and reverse primer ToxB4-*Hin*dIII (5'-CGAAAGCTTCCCTATACCTAATGTAGGG-3'), containing a *Hind*III restriction site (underlined), were used to amplify a 432 bp fragment that included the entire 261 bp *ToxB* ORF. A standard PCR was carried out in a 25 µl reaction volume containing 1 × PCR buffer (Invitrogen), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 100 ng DNA template and 1.0 U Platinum® *Taq* DNA polymerase (Invitrogen). The reaction conditions consisted of an initial denaturation step at 95°C for 5 min; 30 cycles at 94°C for 50 s, 55°C for 50 s, and 72°C for 50 s; and a final extension at 72°C for 2 min. Amplification reactions were carried out in a GeneAmp® PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The amplicons

were resolved on 1% (w/v) agarose gels with the bands of interest excised under UV light, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON, Canada). The purified amplicons were submitted to Macrogen (Rockville, MD, USA) for sequencing using BigDye Terminator cycling conditions on a 3730xl DNA Analyzer (Applied Biosystems, Foster city, CA, USA). After the sequences were confirmed as *ToxB*, the PCR products were digested with *Xho*I and *Hin*dIII (New England Biolabs, Pickering, ON, Canada) for 2 hours at 37°C according to the manufacturer's instructions.

4.2.3. Construction of the transformation vector

The pSilent-1 plasmid developed by Nakayashiki *et al.* (2005), which carries a *hygromycin B phosphotransferase* (*hph*) gene and ampicillin resistance gene cassettes, was obtained from the Fungal Genetics Stock Center (McCluskey *et al.*, 2010). To clone the *ToxB* fragment, pSilent-1 was digested with *Xho*I and *Hin*dIII (New England Biolabs) for 4 hours at 37°C as per the manufacturer's recommendations, and gel purified with a QIAquick Gel Extraction Kit (Qiagen) as above. A 150 ng aliquot of *ToxB* amplicon was then ligated into the 5' *Xho*I-*Hin*dIII cloning site of the digested pSilent-1 vector (50 ng) with T4 DNA ligase, using a Rapid DNA Ligation Kit (Roche, Laval, QC, Canada) as per the manufacturer's instructions; the resulting transformation vector was termed pSilent-ToxB2.

Following ligation, 2 µl of pSilent-ToxB2 was mixed with 50 µl of DH5α chemically competent *Escherichia coli* (Invitrogen) cells, which were transformed via the heat shock method as described in Kim and Strelkov (2007). The pSilent-

ToxB2 transformed *E. coli* cells were spread onto Luria-Bertani [LB (Miller)] agar plates containing ampicillin (100 μg/mL) and incubated overnight at 37°C. To select positive pSilent-ToxB2 plasmid containing cells, 12 emerging bacterial colonies were randomly selected and analyzed by colony PCR (Güssow and Clackson, 1989) with the ToxB6-*Xho*I/ToxB4-*Hin*dIII primer set as described above. Positive clones were then sub-cultured in liquid LB medium containing ampicillin (100 μg/mL), and pSilent-ToxB2 plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was resolved on 1% (w/v) agarose gels and the band confirmed to be of the expected size for the pSilent-ToxB2 plasmid; the DNA was excised and purified from multiple gels with a QIAquick Gel Extraction Kit (Qiagen) as above, and sequenced (Macrogen) to ensure correct *ToxB* insertion into the transformation vector.

4.2.4. Protoplast generation

Protoplasts for fungal transformation were produced from mycelium of the *P. teres* isolate Dillon as described by Aragona and Porta-Puglia (1993) with some modifications. To harvest the mycelium, 2 mL of sterile 0.25 × potato dextrose broth (PDB) was added to V8 agar cultures grown as described above, with mycelium of *P. teres* harvested immediately afterwards by scraping the surface of the cultures with a sterile surgical blade. The harvested hyphae were then homogenized until uniformly fragmented with a glass tissue homogenizer (Pyrex glass, Corning Inc., NY, USA) and 10 mL of this suspension was inoculated into 500 mL of 0.25 × PBD, which was incubated at RT in darkness on a rotary shaker with gentle agitation for 18 hours. The young mycelium was

harvested by filtering the suspension through sterile mira-cloth and homogenized once more, with another 10 mL aliquot used to inoculate a fresh volume (500 mL) of 0.25 × PBD.

After 18 hours of incubation at RT, young mycelium was filtered as above and washed with sterile distilled water to remove the PDB. After removing excess water, 3 g of mycelium was transferred to a sterile Petri dish (5 cm in diameter) and digested with 8 mL of a filter-sterilized cell-wall degrading enzyme mix solution containing 40 mg of driselase (Sigma-Aldrich, St. Louis, MO), 50 mg of yatalase (Takara Mirus Bio, Madison, WI) and 160 mg of lysing enzymes (Sigma-Aldrich) in 1.2 M $MgSO_4$, 0.15 M sorbitol (Sigma-Aldrich) and 20 mM potassium phosphate osmotic buffer, pH 5.8. The suspension was incubated in darkness with gentle agitation at 33°C for 3 h. The protoplasts were separated from mycelial debris by filtering the suspension through a sterile nylon mesh, and the remaining mycelia on the filter were rinsed twice to wash with a total volume of 40 mL filter-sterilized STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$ and10 mM Tris-HCl, pH 7.5) to collect any protoplasts that may have adhered to the mycelium or filter. The protoplasts were collected by centrifugation at 4°C for 10 min at 3000 × g, with the resulting pellet was re-suspended in 100 µL of filter-sterilized STC buffer. The final protoplast concentration was adjusted to 1×10^7 protoplasts/mL in a sterile buffer containing STC, polyethylene glycol (PEG) solution [40% (w/v) PEG 4000, 50 mM $CaCl_2$ and 50 mM Tris, pH 7.5] and dimethyl sulfoxide (Sigma-Aldrich) [80:20:1], and 100 µL aliquots were

immediately used for fungal transformation as described below, or stored at -80^{o}C until needed.

4.2.5. Transformation of *Pyrenophora teres*

Fungal transformation was conducted as previously described (Ciuffetti *et al.*, 1997) with some modifications. Briefly, 100 µL of the *P. teres* protoplast suspension prepared above was mixed with 3 µg of pSilent-ToxB2 vector and 1 µL of 0.1 M spermidine (Fluka, Oakville, ON, Canada), and incubated on ice for 1 h. One mL of 40% PEG 4000 solution was added to the suspension, gently mixed, and incubated at RT for 20 min. A 4 mL aliquot of molten regeneration medium (RM; 1.2 M sorbitol, 0.1% yeast extract, 0.1% casein hydrolysate, and 0.8% agar) was added and the resulting protoplast mix was poured on top of 9 cm-diameter Petri dishes containing 20 ml of solidified RM. The Petri dishes were incubated in darkness at RT for 24 h, following which the protoplasts were overlaid with 5 mL of 0.8% molten RM containing hygromycin B at a final concentration of 150 µg/mL.

4.2.6. Transformant selection

After 7 days incubation in darkness at RT, colonies of hygromycin-resistant transformants were individually transferred onto 9 cm-diameter Petri dishes filled with V8 agar amended with 200 µg/ml hygromycin B, and were consecutively selected on this medium at least five additional times by hyphal tip transfer. Protoplasts that did not possess a copy of the *hph* gene could not grow on hygromycin B containing selection medium (negative control), while these cells could grow on normal V8-agar medium without hygromycin B (positive control).

To confirm successful ectopic integration of *hph* and *ToxB* into the genome of *ToxB*-transformed strains of *P. teres*, genomic DNA was extracted from fresh mycelium of each transformant as above and subjected to PCR with the *hph*-specific primers HPHF (5'-GAATTCAGCGAGAGCCTGAC-3'; forward primer) and HPHR (5'-GATGTTGGCGACCTCGTATT-3'; reverse primer) and the *ToxB*-specific primers ToxB6-*Xho*I and ToxB4-*Hin*dIII. PCR conditions were as described above. DNA from the wild-type *P. teres* isolate Dillon and the *P. tritici-repentis* isolate Alg3-24 was also included in the analyses as controls. All statistical analyses were conducted using SAS software (version 9.2; SAS Institute, Cary, NC, USA).

4.2.7. Southern blot analysis

For Southern blot analysis, genomic DNA was extracted from 40 mg of lyophilized mycelium using a Wizard® Genomic DNA Extraction Kit (Promega) as described above. Fifteen µg of the genomic DNA was then digested with the restriction endonuclease *Bam*H1, which does not cut within the sequence of *ToxB* ORF, and separated by electrophoresis on a 1% agarose gel. The *ToxB* probe was a 245 bp cDNA of *ToxB* ORF from *P. tritici-repentis* race 5 isolate Alg3-24 (GenBank accession number AF483831), which was amplified using the ToxB1 (5'-GACTACCATGCTACTTGCTGTG-3') and ToxB2 (5'-AACAACGTCCTCCACTTTGC-3') primers by a standard PCR with the same reaction conditions as described above. The probe was labeled with digoxigenin (DIG) via the random primed labeling method for the nonradioactive DIG system

(Roche). DNA transfer, fixation, hybridization and detection were also carried out using the DIG system according to the manufacturer's recommendations (Roche).

4.2.8. Western blot analysis

To confirm production of Ptr ToxB, the *P. teres* transformants, as well as the wild-type strains of *P. teres* and *P. tritici-repentis*, were cultured on Fries' medium (amended with 0.1% yeast extract) (Dhingra and Sinclair, 1985) containing hygromycin B (150 µg/mL) as previously described (Strelkov *et al.*, 2002). The culture filtrates were collected by vacuum filtration through Whatman No. 1 filter paper followed by filtration through 0.45 µm cellulose nitrate membrane filters, and concentrated by freeze-drying. The concentrated culture filtrates were then resuspended in 20 mM sodium acetate buffer (pH 4.6) and impurities were removed by centrifugation. The soluble fractions of the redissolved samples were then dialyzed against water overnight and concentrated again by freeze-drying.

The presence and relative abundance of Ptr ToxB was assessed in the concentrated culture filtrates via Western blot analysis with rabbit polyclonal antibodies raised against Ptr ToxB, using 3 µg of total soluble protein per sample as per previously published protocols (Cao *et al.*, 2009; Kim and Strelkov, 2007). Ptr ToxB band intensities were quantified with ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA) and are expressed relative to the intensity of the toxin band in isolate Alg3-24 of *P. tritici-repentis*.

4.2.9. Evaluation of growth rates

To compare fungal growth rates, the *P. teres* transformants were incubated on hygromycin-containing V8 agar medium for 6 days at RT in darkness. The wild-type *P. teres* isolate Dillon was grown as a control. Colony diameters were measured in two directions at right angles to each other and then averaged over the number of replications. Growth rates were recorded from five independent measurements.

4.2.10. Bioassays

The pathogenicity of the *P. teres* wild-type and transformed strains, as well as isolate Alg3-24 of *P. tritici-repentis*, was evaluated on two hexaploid wheat genotypes (line 6B662 and 'Salamouni'), one general purpose barley cultivar Rivers and one malting barley cultivar Harrington. Wheat line 6B662 is susceptible to Alg3-24 and sensitive to Ptr ToxB, while 'Salamouni' is resistant to Alg3-24 and insensitive to the toxin. The barley genotype 'Rivers' is resistant to the spot form of net blotch, whereas 'Harrington' is susceptible to this disease. Five to six seeds of each wheat or barley genotype were sown in 12.7 cm-diameter plastic pots containing a commercial potting medium (Sunshine LA4 Aggregate Plus Mix, Sun Gro Horticulture Canada Ltd., Seba Beach, AB). The seedlings were maintained in a growth cabinet set at 21/18ºC (day/night) with a 16 hour photoperiod (180 μmol photons m^{-2} s^{-1}), and were watered and fertilized as needed.

At the three leaf stage (14 days), the seedlings were inoculated with a suspension of 3×10^3 conidia/ml, to which 10 drops of polyoxyethylene sorbitol

monolaurate (Tween 20) per liter had been added. The spore suspension was applied to the leaves with an atomizer (Fisher Scientific, Ottawa, ON) until runoff. After inoculation, the seedlings were transferred to a misting chamber, where they were kept in darkness under high relative humidity (≥ 95%) for the next 24 h. The plants were then transferred to a growth cabinet and kept at 21/18 ºC (day/night) with a 16 hour photoperiod (180 µmol photons m^{-2} s^{-1}). The bioassay experiments were independently repeated three times with three replicates (pots) per treatment. The second and third leaves were collected 7 days after inoculation and randomly chosen sets of 8-12 leaves were scanned on a flatbed scanner (Epson Perfection 2480 Photo), with the total leaf and lesion areas measured using Assess 2.0 Image Analysis Software (Lamari, 2008).

4.2.11. Fungal recovery from inoculated leaves

Two weeks after inoculation, leaf samples were collected to recover the inoculated strains of *P. teres* from the wheat leaves. Leaf segments about 3 cm in length were surface-sterilized for 15 s in 70% (v/v) ethanol and 30 s in 10% (v/v) bleach (6.15% sodium hypochlorite), and then rinsed three times in sterile water. After being allowed to air dry, the leaf segments were placed on 9 cm-diameter Petri dishes containing V8 agar with ampicillin (100 µg/mL) and hygromycin (200 µg/mL) (for leaves inoculated with *P. teres* transformants), or with only ampicillin (100 µg/mL) (for leaves inoculated with the wild-type *P. teres* isolate). The Petri dishes were incubated for 5 days at RT under a 12 h photoperiod as above. Hyphal tips from the emerging mycelia were transferred to fresh 20% V8 agar plates containing ampicillin (100 µg/mL) and hygromycin (200 µg/mL) or

ampicillin (100 µg/mL) only, and were incubated for 10 days in darkness at RT. Genomic DNA was then extracted from the colonies as described above. To confirm that the recovered isolates were indeed *P. teres* f. *maculata*, a PCR was performed with the *P. teres* f. *maculata*-specific primers PTM-F (5'-TGCTGAAGCGTAAGTTTC-3') and PTM-R (5'-ATGATGGAAAAGTAATTTGTG-3'), according to the method of Williams *et al*. (2001). To determine whether or not the re-isolated strains of *P. teres* contained the *ToxB* gene, the DNA from each sample was subjected to PCR with the *ToxB*-specific primers ToxB6-*Xho*I and ToxB4-*Hin*dIII.

4.3. Results

4.3.1. Transformant selection and screening

Transformation of the 'wild-type' *ToxB* gene from race 5 isolate Alg3-24 of *P. tritici-repentis* into isolate Dillon of *P. teres* f. *maculata* was achieved by PEG-mediated fungal transformation with the pSilent-ToxB2 vector encoding the *hph* gene as a selection marker. Approximately 100 emerging colonies on hygromycin B-containing V8 agar plates were selected by hyphal tip transfer and screened five consecutive times on the same medium. To screen out false positives, the transformants were tested for the presence of both the *hph* and *ToxB* genes by PCR with *hph*- and *ToxB*-specific primers; only transformants carrying both genes were selected for further analyses. No bands were detected in the wild-type *P. teres* isolate Dillon, which was included as a negative control. Finally, a total of three fast growing transformants denoted as PT24, PT51 and PT74 were

selected, and a single-spore from each was isolated and cultured for further evaluation.

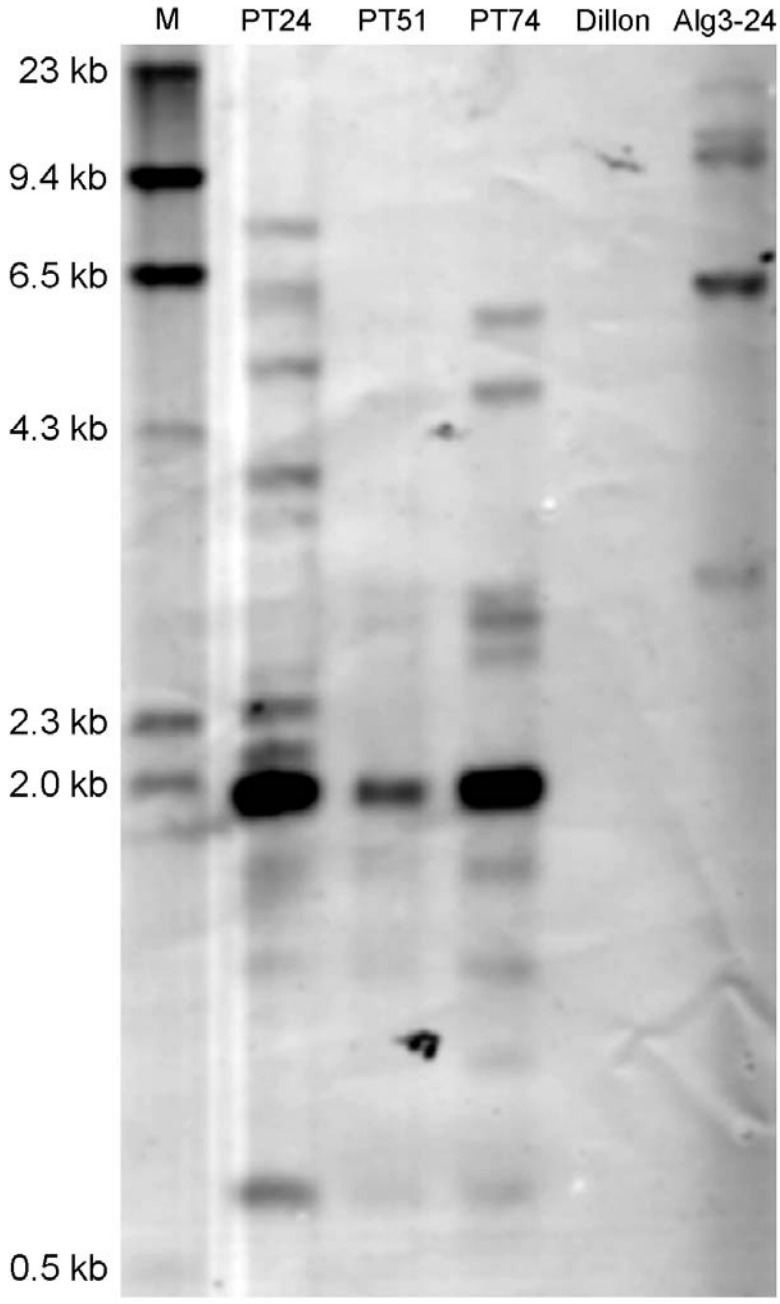
4.3.2. Southern blot analysis

A Southern blot analysis with DIG-labeled *ToxB* probe was used to detect the integration of *ToxB* into the genome of the *P. teres* transformants PT24, PT51 and PT74 (Fig. 4-1). No band was observed in Southern blots of the wild-type *P. teres* isolate Dillon, while multiple bands were observed in genomic DNA of the wild-type race 5 isolate Alg3-24 of *P. tritici-repentis*. A strong band was also observed in DNA of the transformants PT24, PT51 and PT74, just below the 2.0 kb maker, representing the 1,963 bp transcriptional unit of the *ToxB* gene from pSilent-ToxB2 (which consists of a 1,531 bp fragment from the pSilent1 vector and 432 bp from the cloned *ToxB* gene).

4.3.3. Western blot analysis

The amount of Ptr ToxB produced by the transformed *P. teres* strains, the wild-type *P. teres* isolate Dillon, and the *P. tritici-repentis* isolate Alg3-24 was assessed by Western blot analysis of concentrated culture filtrates with polyclonal antibodies raised against His-tagged Ptr ToxB (Cao *et al.*, 2009). A band with a mass of approximately 6 kDa, corresponding to the expected size of Ptr ToxB, was visible in culture filtrates of Alg3-24 and the *P. teres* transformants PT24, PT51 and PT74, but was absent from culture filtrates of the wild-type *P. teres* isolate Dillon (Fig. 4-2). The intensity of the toxin band, however, was significantly ($p < 0.05$) lower in the *P. teres* transformants than in Alg3-24, suggesting significantly reduced production of Ptr ToxB in the former. In culture

Figure 4-1. Southern blot analysis of genomic DNA from *ToxB*-transformed strains of *Pyrenophora teres* f. *maculata*, the wild-type *P. teres* isolate Dillon and race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis,* using a digoxigenin-labeled *ToxB* probe. Fifteen µg of BamH1-digested genomic DNA of each fungal strain was loaded in each lane. Lanes show (left to right): DNA Molecular Weight Marker II (200 ng; Roche) (M); *P. teres* transformants PT24; PT51; PT74; lane 5, *P. teres* isolate Dillon; and *P. tritici-repentis* isolate Alg3-24. Multiple integration of the *ToxB* gene into genomic DNA was detected in the *ToxB*-transformed strains of *P. teres*, while no band was observed in the wild-type *P. teres* isolate (Dillon). Race 5 isolate Alg3-24 of *P. tritici-repentis*, which served as the source of the 'wild-type' *ToxB* gene, was included as a positive control and contained multiple *ToxB* bands.


M
PT24
PT51
PT74
Dillon
Alg3-24
23 kb
9.4 kb
6.5 kb
4.3 kb
2.3 kb
2.0 kb
0.5 kb

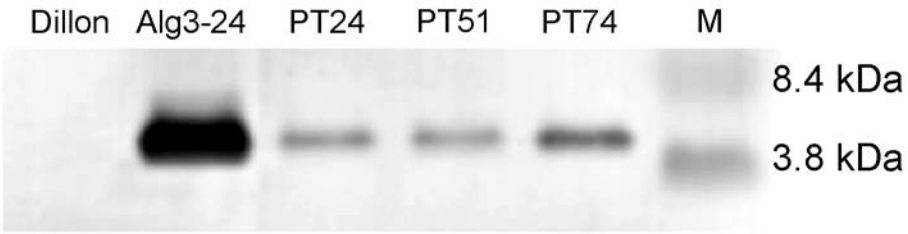


Figure 4-2. Western blot analysis of culture filtrates from the wild-type isolate Dillon of *Pyrenophora teres* f. *maculata*, race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*, and three *ToxB*-transformed strains of *P. teres*, with polyclonal antibodies specific to Ptr ToxB. Three µg of total protein from 21-day-old concentrated culture filtrates of each fungal strain was loaded per lane. Lanes show (left to right): *P. teres* isolate Dillon; *P. tritici-repentis* isolate Alg3-24; *P. teres* transformants PT24; PT51; PT74; and Kaleidoscope Polypeptide Standards (Bio-Rad). Note the presence of a band corresponding to Ptr ToxB in PT24-PT74, and its absence in Dillon.

filtrates of PT74, the intensity of the Ptr ToxB band was 23% of that in Alg3-24; in culture filtrates of PT24 and PT51, the intensity of the toxin band was only 14% and 12%, respectively, of that in the *P. tritici-repentis* isolate.

4.3.4. Colony growth

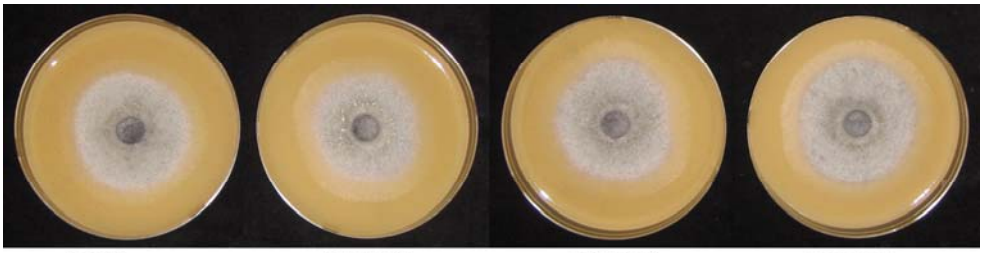
The colony morphology of the *P. teres* transformants PT24, PT51 and PT74 on hygromycin-containing V8 agar medium was similar to that of the wild-type isolate Dillon (Fig. 4-3A). Similarly, the growth rates of the transformed strains were not significantly ($p < 0.05$) different from the wild-type isolate, with all colonies having an average diameter ranging from 6.17 to 6.24 cm after 6 days of incubation in darkness at RT (Fig. 4-3B).

4.3.5. Bioassays

The *P. teres* transformants PT24, PT51 and PT74 and the wild-type isolate Dillon were tested for their virulence on the Ptr ToxB-sensitive wheat line 6B662 and the Ptr ToxB-insensitive wheat cv. Salamouni, as well as on the barley cvs. Harrington and Rivers. Dillon produced only trace levels of minute flecks when inoculated onto the wheat line 6B662 and cv. Salamouni (Fig. 4-4). The transformed *P. teres* strains caused the development of larger necrotic flecks on 6B662, but like the wild-type isolate, caused virtually no visible symptoms on 'Salamouni' (Fig. 4-4). The necrotic flecks on 6B662 covered 1.29% ± 0.25% (average ± standard error) of the total leaf surface 7 days after inoculation with PT24, 1.49% ± 0.02% of the leaf surface after inoculation with PT51, and 1.89% ± 0.09% of the leaf surface after inoculation with PT74. This amount of necrotic flecking was significantly ($p < 0.05$) greater than that caused by the same strains

Figure 4-3. Growth of the wild-type isolate Dillon of *Pyrenophora teres* f. *maculata* and the hygromycin-resistant *ToxB*-transformed strains of the fungus. (A) Morphology of all tested strains. (B) Growth of fungal colonies expressed as colony diameter in centimeters. Cultures were maintained in 9 cm-diameter Petri dishes filled with V8 agar medium for 6 days at RT in darkness, and colony diameter was measured at two points perpendicular to each other. The mean of five replications (cultures) is shown, with bars representing the standard error of the mean.

A


Dillon
PT24
PT51
PT74

B

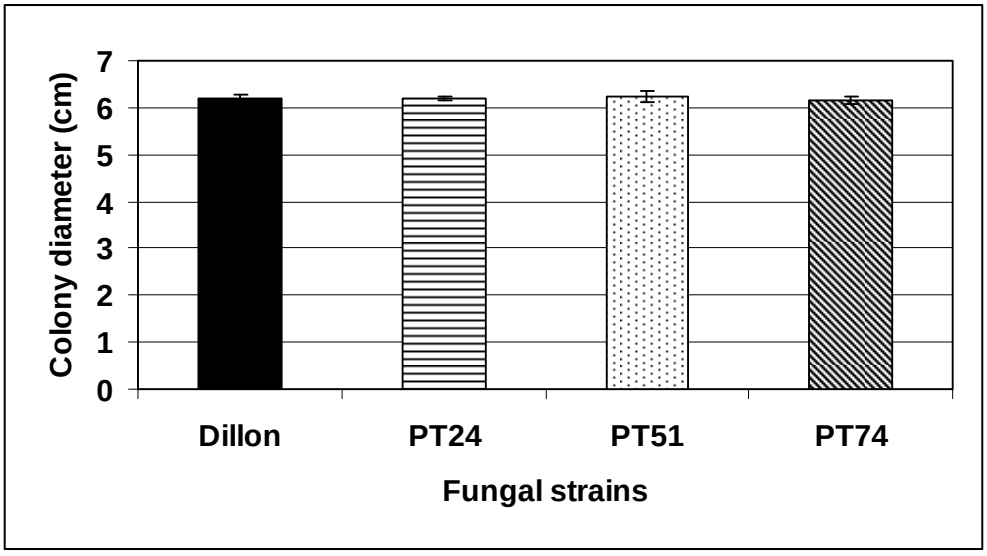
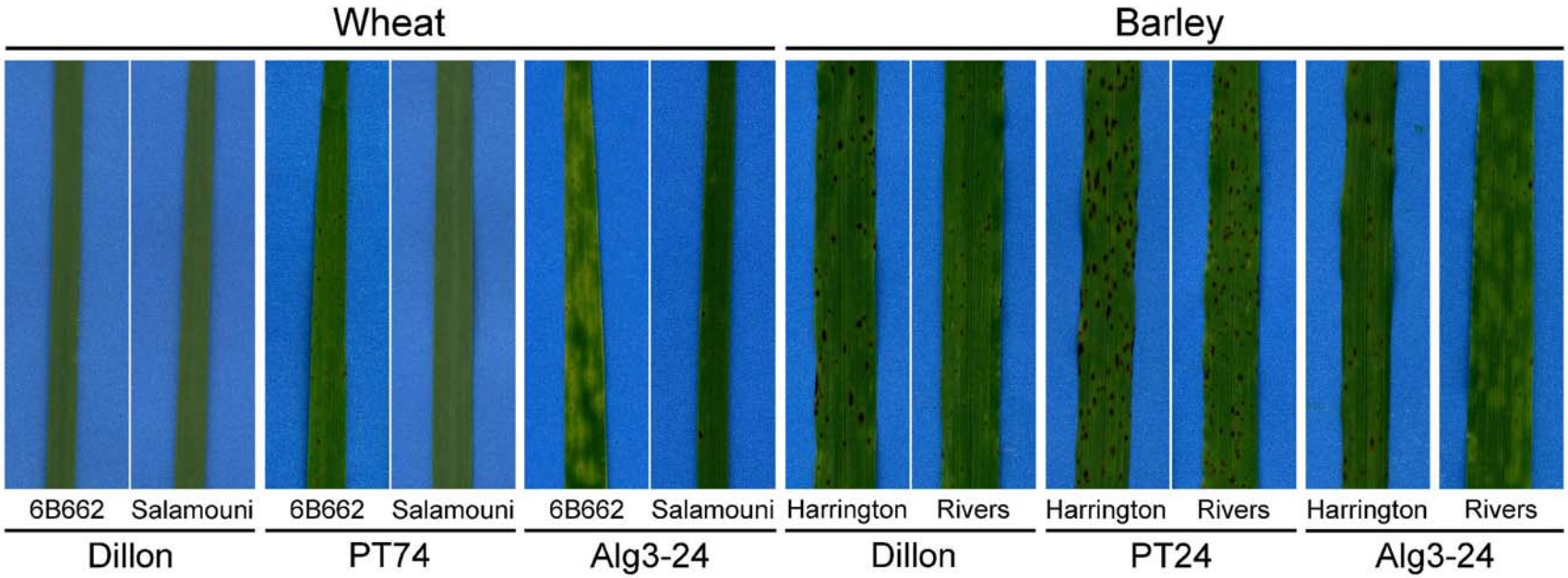

Colony diameter (cm)
0
1
2
3
4
5
6
7
Dillon
PT24
PT51
PT74
Fungal strains

Figure 4-4. Reactions of selected wheat and barley genotypes to inoculation with the wild-type isolate Dillon and the *ToxB*-transformed strains PT74 or PT24 of *Pyrenophora teres* f. *maculata*,, and the race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*. Leaves are shown 7 days after inoculation with the respective strains. Wheat line 6B662 is sensitive to Ptr ToxB, while cv. Salamouni is insensitive to the toxin. Barley genotype cv. Harrington is susceptible to net blotch, while cv. Rivers is resistant to the disease. Similar results were obtained with all *ToxB*-transformed strains on both wheat and barley.


Wheat
Barley
6B662
Salamouni
6B662
Salamouni
6B662
Salamouni
Harrington
Rivers
Harrington
Rivers
Harrington
Rivers
Dillon
PT74
Alg3-24
Dillon
PT24
Alg3-24

on ‘Salamouni’ (0.02% ± 0.01% for all three transformants) and by the wild-type *P. teres* on 6B662 (0.05% ± 0.01%) and ‘Salamouni’ (0.03% ± 0.01%). As expected, inoculation of the wheat genotypes with race 5 isolate Alg3-24 of *P. tritici-repentis* resulted in a typical susceptible (chlorotic) reaction on 6B662 and a highly resistant reaction on ‘Salamouni’ (Fig. 4-4).

On the net blotch susceptible barley cv. Harrington, all *P. teres* transformants caused significantly ($p < 0.05$) more symptoms than the wild-type isolate Dillon (Fig. 4-4). While dark-brown lesions covered an average of 13.7% ± 0.4% of the leaf surface on ‘Harrington’ 7 days after inoculation with Dillon, inoculation with the transformants PT24, PT51 or PT74 resulted in lesions on 29.1% ± 0.01%, 25.7% ± 2.8% and 28% ± 0.14% of the leaf surface, respectively (Fig. 4-4). Similarly, the transformed *P. teres* strains caused significantly ($p < 0.05$) more symptoms than the wild-type isolate on the net blotch resistant barley cv. Rivers; 3.59 % ± 0.8 % of the leaf surface on this cultivar was covered with brown lesions 7 days after inoculation with Dillon, while lesions covered between 12.9% ± 0.21% to 14.3% ± 0.62% of the total leaf area following inoculation with PT24, PT51 or PT74. Inoculation of ‘Harrington’ with *P. tritici-repentis* isolate Alg3-24 caused the development of brown spots on 9.77 % ± 0.73% of the leaf surface, a value significantly lower ($p < 0.05$) than that caused by the wild-type and transformed *P. teres* strains. Interestingly, on the net blotch resistant barley cv. Rivers, Alg3-24 caused the development of chlorotic lesions similar to those produced on wheat line 6B662, which covered 25% ± 0.33% of the total leaf area (Fig. 4-4).

4.3.6. Recovery of fungal isolates from inoculated leaves

In order to assess survival of the transformed and wild-type *P. teres* strains on the Ptr ToxB-sensitive wheat line 6B662, leaves were collected 2 weeks after inoculation and a fungal recovery assay was conducted. After surface sterilization of the leaves, cultures of *P. teres* could be recovered at high-frequency on hygromycin-containing V8 agar medium from tissue that had been inoculated with PT24, PT51 or PT74. In contrast, *P. teres* cultures were recovered only at very low frequencies on V8 agar medium from tissue inoculated with the wild-type isolate Dillon. To confirm that the isolates recovered from the leaves were indeed *P. teres* and contained the *ToxB* gene, total genomic DNA was extracted from the fungal cultures and subjected to PCR with primers specific for *P. teres* f. *maculata* (Williams *et al*., 2001) and *ToxB*. A *P. teres* f. *maculata*-specific band could be amplified from the re-isolated *P. teres* transformants and the wild-type isolate Dillon, but not from the negative control (Fig. 4-5A). An amplicon of the expected size could also be obtained with the *ToxB*-specific primers from the transformed *P. teres* strains, but not from cultures of the re-isolated wild-type isolate (Fig. 4-5B).

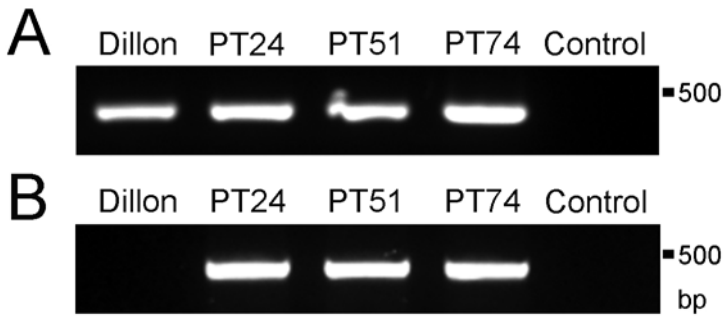


Figure 4-5. PCR detection of *P. teres* f. *maculata* (A) and the *ToxB* gene (B) in genomic DNA of *Pyrenophora teres* f. *maculata* strains recovered from leaves of wheat line 6B662 14 days after inoculation. In both panels, the lanes (left to right) show: wild-type *P. teres* isolate Dillon; *P. teres* transformants PT24; PT51; PT74; and negative control. **A**, when *P. teres* f. *maculata*-specific primers were used for the PCR, an amplicon of the expected size was obtained from both the wild-type *P. teres* and the *ToxB*-transformed *P. teres* strains. **B**, when the *ToxB*-specific primers were used, an amplicon was obtained only from the *ToxB*-transformed strains of *P. teres*.

4.4. Discussion

While there have been no reports of the production of ToxB-like proteins by *P. teres*, some isolates of this fungus have been shown to possess a *ToxB* homolog (Andrie *et al.*, 2008). The role of this homolog in *P. teres*, however, is unknown. Given the importance of Ptr ToxB in mediating the interaction between wheat and *P. tritici-repentis*, we hypothesized that the acquisition of Ptr ToxB-producing ability by *P. teres* could be a sufficient condition for pathogenicity by this pathogen on wheat. Indeed, while *P. teres* is primarily a barley pathogen (Mathre, 1997), some isolates of the fungus have been also found to infect wheat and a wide range of grasses (Liu *et al.*, 2011; Mikhailova *et al.*, 2010; Tóth *et al.*, 2008; Turkington *et al.*, 2002). This ability to infect non-barley hosts could be augmented (in theory) by the production of necrotrophic effectors such as Ptr ToxB. In fact, the interspecific transfer of the *ToxA* gene from *Stagonospora nodorum* (Berk.) Castell. & Germano, which encodes the host-specific toxin ToxA, has been implicated in the emergence of *P. tritici-repentis* as a major pathogen of wheat (Friesen *et al.*, 2006).

To test the hypothesis that production of Ptr ToxB could convert *P. teres* into a pathogen of wheat, we transformed an isolate of *P. teres* f. *maculata* lacking *ToxB* or any of its homologs with a wild-type copy of the gene from *P. tritici-repentis*. The successful introduction of the *ToxB* gene into the genome of *P. teres* was confirmed by PCR and Southern blotting analysis, while production of the Ptr ToxB protein was confirmed by Western blotting (Fig. 4-1 and 4-2). The transformed *P. teres* strains were able to produce significantly more severe

symptoms of net blotch than the wild-type isolate on barley cultivars resistant or susceptible to the disease (Fig. 4-4). On the Ptr ToxB-sensitive wheat line 6B662, however, the transformants caused only necrotic flecking, despite their ability to produce Ptr ToxB. While this flecking represented an increase in symptoms relative to those induced by the wild-type isolate, which caused virtually no visible symptoms on 6B662, it did not resemble a compatible interaction. Instead, the symptoms induced by the transformed strains of *P. teres* more closely resembled the flecking caused by non-pathogenic race 4 isolates of *P. tritici-repentis*. The enhanced rates of recovery of the transformants from inoculated leaves of wheat line 6B662 (Fig. 4-5) also indicated their continued persistence in the resistant host tissue, as has been observed with *P. tritici-repentis* race 4 (Amaike *et al.*, 2008).

Several independent studies have suggested that Ptr ToxB acts in a dosage-dependent manner, with gene copy number (Amaike *et al.*, 2008; Ciuffetti *et al.*, 2010), transcript quantity (Amaike *et al.*, 2008) and the amount of toxin produced (Aboukhaddour *et al.*, 2012) all correlated with virulence or the degree of fungal pathogenicity on Ptr ToxB-sensitive hosts. Thus, a race 5 isolate of *P. tritici-repentis* that was a strong producer of Ptr ToxB caused significantly more disease than another race 5 isolate that produced lower levels of the toxin (Amaike *et al.*, 2008). Similarly, the virulence of race 5 strains in which *ToxB* had been silenced was correlated with the degree of silencing (Aboukhaddour *et al.*, 2012), while the virulence of race 4 strains that had been transformed with *ToxB* was correlated with gene copy number (Ciuffetti *et al.*, 2010). These observations

suggest that the inability of the transformed *P. teres* strains to cause significant levels of disease on the Ptr ToxB-sensitive wheat line 6B662 could be explained by their relatively low level of toxin expression, since the Ptr ToxB bands produced by the transformants had an intensity only 12 to 23% of that produced by the pathogenic race 5 isolate of *P. tritici-repentis* (Fig. 4-2). This suggestion is also supported by the recent finding that while the *P. tritici-repentis* sister species *Pyrenophora bromi* (Died.) Drechsler expresses a *ToxB* homolog *in planta*, the level of expression appears to be insufficient to overcome the resistance response in a ToxB-sensitive wheat (Andrie and Ciuffetti, 2011).

Nevertheless, it is likely that other factors, perhaps related to the basic compatibility between *P. teres* and wheat, may have also contributed to the limited symptom development induced by the transformants on line 6B662. The *P. teres* strain PT74, which in culture produced a Ptr ToxB toxin band with an intensity 23% of that produced by the race 5 isolate Alg3-24 of *P. tritici-repentis* (Fig. 4-2), caused development of lesions that covered only 1.89% ± 0.09% of the leaf surface on 6B662. In contrast, in a recent study in which *ToxB* was silenced in the same isolate of *P. tritici-repentis*, a silenced strain was obtained that also produced a toxin band with an intensity of only 23%, yet was able to cause chlorotic lesions that covered 17% of the leaves of the same host genotype (Aboukhaddour *et al.*, 2012). It has been reported that *P. teres* f. *maculata* initially grows biotrophically before switching to necrotrophic growth in the mesophyll, and that infection of host tissue occurs more slowly than with *P. teres* f. *teres*, which grows only as a necrotroph (Lightfoot and Able, 2010). This initial phase of biotrophic growth

may have also contributed to the limited symptoms caused by the *P. teres* f. *maculata* transformants on wheat line 6B662.

In a somewhat unexpected result, the *P. tritici-repentis* isolate Alg3-24 was found to be pathogenic on the barley cv. Rivers, inducing chlorosis symptoms similar to those produced on the Ptr ToxB-sensitive wheat line 6B662 (Fig. 4-4). This observation suggests that 'Rivers' carries the same toxin-sensitivity gene, *Tsc2*, which has been reported in Ptr ToxB-sensitive wheat genotypes (Abeysekara *et al.*, 2010; Friesen and Faris, 2004). This hypothesis will have to be confirmed by infiltrating the leaves of this and other barley cultivars with purified Ptr ToxB, in order to fully assess sensitivity to the toxin. However, if some barley genotypes are indeed sensitive to Ptr ToxB, there might be increased potential for *P. tritici-repentis* to become a problem in barley production. Moreover, *P. teres* isolates that possess a *ToxB* homolog would also presumably have an advantage when infecting such hosts.

The reason for the significantly increased virulence of the *P. teres* transformants on the two barley cultivars tested is not immediately clear. On the net blotch susceptible barley cv. Harrington, the transformants caused twice as much disease as the wild-type, while on the resistant cv. Rivers they caused approximately four times as much disease (Fig. 4-4). This effect did not seem related to the typical Ptr ToxB-induced chlorosis that is seen on toxin-sensitive wheat, since the lesions associated with the increased levels of disease resembled the symptoms of the spot form of net blotch. It has been suggested that Ptr ToxB proteins may have other role(s) in the basic parasitic ability of *P. tritici-repentis*,

perhaps related to pathogenic fitness and/or pre-colonization processes (Amaike *et al.*, 2008). Additional role(s) for Ptr ToxB or Ptr ToxB-like proteins in fungal pathogenicity appears to be supported by the identification of *ToxB* homologs in *P. bromi*, *P. teres* and other ascomycetes that attack non-wheat hosts (Andrie *et al.*, 2008), as well as by observed correlations between *ToxB*/Ptr ToxB expression and the number of appressoria formed on leaf tissue (Aboukhaddour *et al.*, 2012; Amaike *et al.*, 2008). It is possible, therefore, that the increased levels of disease caused on barley by the *P. teres ToxB* transformants reflected these other possible roles of Ptr ToxB. More studies, aimed at comparing the infection process in the transformed and wild-type *P. teres* strains, are underway in order to assess this possibility. It is also important to note that to date, no other virulence or effector genes have been mapped or identified from *P. teres* f. *maculata* (Liu *et al.*, 2011), and that the genome sequence of this pathogen is currently unavailable. Hence, it is also possible that other, as of yet unknown virulence factors may have interacted with the Ptr ToxB produced by the transformants, thereby contributing to increased symptom development.

To our knowledge, this is the first report in which *ToxB* from *P. tritici-repentis* was transformed into another species. Our results suggest that while production of Ptr ToxB in *P. teres* resulted in increased virulence on net blotch resistant and susceptible barley cultivars, it was not sufficient (at least at the levels produced) to cause disease on a toxin-sensitive wheat line. Nonetheless, production of Ptr ToxB by *P. teres* resulted in an interaction phenotype which resembled that of non-pathogenic race 4 isolates of *P. tritici-repentis*, and which

were distinct from that of the wild-type *P. teres* isolate. The increased virulence of the transformed strains on barley, and the shift in interaction phenotype following inoculation onto wheat, suggest an effect of *ToxB* on the parasitic ability of *P. teres*, thereby providing clues as to the role of naturally occurring ToxB-like sequences that have been identified in other isolates of this fungus.

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5. General Discussion

In this dissertation, I presented research intended to improve our understanding of the role and mechanism of action of Ptr ToxB, with three specific objectives: (1) to examine the direct effects of Ptr ToxB on photosynthesis and the host leaf proteome at various time-points prior to the development of visible chlorosis symptoms, (2) to investigate the role of Ptr ToxB as both a pathogenicity and virulence factor via transformation of a Ptr ToxB non-producing isolate of *P. tritici-repentis* with the *ToxB* gene, and (3) to determine whether the acquisition of Ptr ToxB-producing ability can make a non-wheat pathogen (*P. teres*) infectious on wheat. At this stage, it is useful to reflect on the various components of the work, and to identify potential areas for future study to build on the current findings.

5.1. Proteome and photosynthesis changes induced by Ptr ToxB

As part of long term Ptr ToxB mode of action studies, we conducted a comparative analysis of proteome-level changes induced by His-tagged Ptr ToxB and examined the direct effects of Ptr ToxB on photosynthesis in toxin-sensitive leaf tissue. Leaves of the wheat cv. Katepwa were infiltrated with toxin or buffer and changes in the proteome occurring at three time-points prior to the development of visible chlorosis were compared by 2-DE, followed by tandem mass spectrometry to identify differentially abundant proteins (Table 2-1, Fig. 2-2). This study was conducted only with a toxin sensitive wheat genotype because a comparison of the proteomes of two different genotypes (i.e., Ptr ToxB-sensitive and insensitive) would have led to difficulties in assessing which differences

resulted from Ptr ToxB activity as opposed to innate differences between the genotypes. To our knowledge, this is the first study that examined the proteome-level changes induced by Ptr ToxB in wheat. In the tan spot pathosystem, which follows the inverse gene-for-gene or toxin model of host-parasite interactions (Strelkov and Lamari, 2003), it is possible to examine the processes associated with effector-triggered susceptibility (ETS) (Jones and Dangl, 2006) in the absence of the fungus itself.

Ptr ToxB was found to affect the abundance of many proteins involved in energy metabolism, particularly those related to photosynthesis (Fig. 2-3A). The levels of other chloroplast-related proteins were also significantly altered at all time-points (Fig. 2-3B-D), a finding confirmed by Western blot analysis with polyclonal antibodies raised against the thylakoid membrane protein fraction (Fig. 2-5A-B). Levels of the antioxidant enzyme ascorbate peroxidase (APX) significantly increased at 24 h after toxin treatment (Table 2-1). Assays to measure total peroxidase and APX activity provided additional evidence of their up-regulation (Fig. 2-4). Similarly, net photosynthesis, as examined by monitoring leaf CO_2 uptake using an infrared gas analyzer, declined significantly within 12 h of toxin treatment and continued to decline throughout the study (Fig. 2-1), indicating that Ptr ToxB inhibits photosynthesis before chlorosis symptoms develop at 48 to 72 h. An inhibition of photosynthesis would explain the formation of reactive oxygen species (ROS) in toxin-sensitive hosts, as illuminated thylakoid membranes become unable to dissipate the excitation energy normally used to drive this process (Strelkov *et al.*, 1998). Therefore, it

appears that treatment with Ptr ToxB results in damage to the integrity of the chloroplast membranes, likely through the formation of ROS, following the inhibition of photosynthesis.

It is interesting to note that the quantities of three spots matching germin-like protein (GLP), which has been proposed to have a function in pathogen defense in cereals (Breen and Bellgard, 2010; Manosalva *et al.*, 2009), were found to decrease more than five-fold after treatment with the toxin (Table 2-1, Fig. 2-2, 2-5D). It has been reported in numerous studies that GLPs have either superoxide dismutase (SOD) or oxalate oxidase (OXO) activity and glycoprotein properties (reviewed in Davidson *et al.*, 2009). Therefore, we conducted enzyme assays and tested for glycoprotein with a Glycoprotein Detection Kit (Sigma) and 1-D and 2-D gel electrophoresis. Although we confirmed that the GLPs had glycoprotein properties, no significant differences were detected with respect to SOD and OXO activities (data not shown). Nonetheless, additional studies on the impact of Ptr ToxB on GLP are warranted, since the observed decreases in GLP could suggest an inactivation of the host defense response by the toxin. This would be consistent with the 'passive' nature of tan spot resistance, in which disease will develop only if the host is sensitive to the toxin (Strelkov and Lamari. 2003).

5.2. Transformation of the *ToxB* gene into *Pyrenophora tritici-repentis*

It was previously shown that the virulence or degree of pathogenicity of naturally occurring isolates of *P. tritici-repentis* is correlated with *ToxB* copy number, with only two copies found in a weakly virulent Canadian race 5 isolate versus multiple copies in highly virulent isolates of races 5, 6, 7 and 8 (Amaike *et*

al., 2008; Lamari *et al.*, 2003; Strelkov *et al.*, 2002; Strelkov *et al.*, 2006). Similarly, transformation of a race 4 isolate of the fungus with *ToxB* revealed that the higher the *ToxB* copy number, the greater the virulence of the transformants (Ciuffetti *et al.,* 2010). In the current work, Southern blot analysis confirmed the multiple integration of *ToxB* into the genome of selected transformants (Fig. 3-2). However, the gene copy number in the transformed strains did not correlate with symptom severity in the bioassays (Fig. 3-4). This seems to indicate that not all copies of the transformed *ToxB* were expressed and/or translated, a suggestion supported by Western blot analysis for the presence of Ptr ToxB (Fig. 3-3). One transformed strain did not carry *ToxB* but did harbor the *hph* gene (Fig. 3-1), indicating that incomplete integration of the vector pSilent-ToxB1 could occur. Ultimately, however, it is the production of Ptr ToxB by the transformed strains that will determine their virulence, and in the current work, the degree of pathogenicity of the transformants was generally well correlated with the amount of toxin protein that they produced (Fig. 3-3, 3-4). Similarly, a previous study revealed that when *ToxB* was silenced, the virulence of the transformed strains was correlated with the degree of gene silencing (Aboukhaddour *et al.*, 2012). Our current results directly showed that the acquisition of Ptr ToxB-producing ability is a sufficient condition for pathogenicity in *P. tritici-repentis*, and that the toxin mediates compatibility between the fungus and different genotypes of its wheat host.

From a methodological perspective, it appears that *Agrobacterium tumefaciens*-mediated fungal transformation can lead to higher transformation

efficiencies relative to PEG-mediated transformation in many fungi, and has the advantage of using intact cells as the starting material without the need for the generation of protoplasts (Amey *et al.*, 2002; Fitzgerald *et al.*, 2003; Michielse *et al.*, 2005a, 2005b). Nevertheless, PEG-mediated fungal transformation in the presence of calcium ions remains the most commonly used technique because of its technical simplicity and requirement for only commonly available laboratory equipment (Liu and Friesen, 2012). In this study, PEG-mediated fungal transformation resulted in many *ToxB*-transformed strains and high transformation efficiency. Therefore, the transformation method used in this study could be applied to the functional analysis of other pathogenicity or virulence genes in *P. tritici-repentis*.

5.3. Transformation of the *ToxB* gene into *Pyrenophora teres*

Homologs of *ToxB* have been identified as single or multiple copies in a broad range of phytopathogenic ascomycete fungi (Andrie *et al.*, 2008). Although there have been no reports of the production of Ptr ToxB by isolates of *P. teres*, the cause of net blotch of barley, some isolates of this fungus have been shown to carry a *ToxB* homolog (Andrie *et al.*, 2008). The *P. teres* f. *maculata* isolate Dillon used in this study, however, did not possess a copy of *ToxB* or *ToxB*-like sequences (Fig. 4-1, 4-5). In an effort to better understand the possible role of *ToxB* homologs in *P. teres* and other non-pathogens of wheat, we transformed this barley pathogen with the wild-type *ToxB* gene from *P. tritici-repentis*.

Although the expression of *ToxB* in *P. teres* was controlled by the strong constitutive *Aspergillus nidulans trpC* promoter from the pSilent-ToxB2 vector

(Nakayashiki *et al.*, 2005), it is likely that not all integrated copies of the gene were expressed in the transformed fungal strains (as discussed above for *P. tritici-repentis*) (Fig. 4-1, 4-2). Nevertheless, Ptr ToxB protein was produced (Fig. 4-2) and the *P. teres* f. *maculata ToxB* transformants caused significantly more disease on net blotch-susceptible and resistant barley cultivars than did the wild-type isolate (Fig. 4-4). *Pyrenophora teres* f. *maculata* initially grows biotrophically in host tissue before switching to necrotrophic growth, and is therefore considered a hemibiotroph (Lightfoot and Able, 2010). It is possible that the transformed *P. teres* strains, by secreting the necrotrophic effector Ptr ToxB, were able to shorten the transition time needed to switch from hemibiotrophic to necrotrophic growth (i.e., the 'biotrophy-necrotrophy switch'; Bhadauria *et al.*, 2011; Dufresne *et al.*, 2000), resulting in more rapid development of symptoms and increased disease severity. However, while the transformants did exhibit an altered infection phenotype on the Ptr ToxB-sensitive wheat line 6B662, the acquisition of Ptr ToxB-producing ability did not appear, by itself, to be sufficient to make *P. teres* pathogenic on wheat (Fig. 4-4).

Another interesting finding of this study was that the race 5 isolate Alg3-24 of *P. tritici-repentis* could infect the barley cv. Rivers, causing chlorosis symptoms similar to those induced on Ptr ToxB-sensitive wheat (Fig. 4-4). This suggests that 'Rivers' carries the same toxin-sensitivity gene, *Tsc2*, which has been identified in Ptr ToxB-sensitive wheat genotypes (Abeysekara *et al.* 2010; Friesen and Faris, 2004). Recently, Mayer *et al.* (2011) reported that the barley (H) genome shows a mosaic of structural similarity to the A, B, and D

subgenomes of common wheat, suggesting that *Tsc2* or similar *ToxB*-sensitivity genes may be present in some barley genotypes. However, additional studies are needed to test the sensitivity of 'Rivers' to different concentrations of Ptr ToxB, and to evaluate whether toxin-sensitivity and susceptibility to isolate Alg3-24 of *P. tritici-repentis* co-segregate. Regardless of whether the same or a different form of *Tsc2* is present in the barley cv. Rivers, or whether other sensitivity genes are involved, the pathogenicity of Alg3-24 on this cultivar suggests that some strains of *P. tritici-repentis* have the potential to become parasites of barley.

5.4. Future Studies

While this thesis has contributed to our understanding of the tan spot pathosystem, and in particular the role of Ptr ToxB, there is much that remains unknown, and hence there is an opportunity for future studies. Among these, one line of research could involve toxin localization studies with confocal microscopy using Ptr ToxB tagged with green fluorescent protein (GFP). It has been shown that in the case of Ptr ToxA, the toxin is internalized in cells of sensitive but not insensitive host plants (Manning and Ciuffetti, 2005). The same might be happening with Ptr ToxB. Treated tissues could be examined microscopically to determine if the toxin is associated with or accumulates in particular organelles or sub-cellular compartments. Of particular interest is whether a difference would be observed in terms of the fate of Ptr ToxB in toxin-sensitive and insensitive cells. This will provide more specific information on the site of Ptr ToxB action.

In addition to *in planta* studies to determine the subcellular localization of Ptr ToxB, other approaches could be employed to identify the exact target of toxin

action. Currently, no information is available regarding which (if any) host protein(s) interacts directly with Ptr ToxB. Thus, to identify a possible target(s) of Ptr ToxB in toxin-sensitive cells, protein-protein interaction assays, such as the yeast two-hybrid assay, could be carried out. The yeast-two hybrid assay already has been successfully applied in the tan spot pathosystem, in order to identify target proteins for the necrosis-inducing toxin Ptr ToxA (Manning *et al.*, 2007; Tai *et al.*, 2007). With regards to the identification of targets for Ptr ToxB, the commercially available Gal4 system (Stratagene, La Jolla, CA, U.S.A.) could be used to generate a Ptr ToxB bait construct with a DNA-binding domain and a prey construct library with a Gal4 activation domain, as previously reported for Ptr ToxA (Manning et al., 2007; Tai et al., 2007). In the Gal4 system, the end result of an interaction between two proteins is a change in color to blue in X-gal (a chromogenic substrate)-containing medium, as a result of galactosidase activity. To verify that the Ptr ToxB-fusion protein is actually expressed inside the yeast, Western blot analysis with polyclonal antibodies against Ptr ToxB would have to be carried out before proceeding to the screening stage of the assay. Inclusion of appropriate controls, exclusion of false positives and a test for auto-activation (i.e., whether or not the Ptr ToxB bait itself activates transcription) would also be necessary prerequisites for successful application of this approach. Nevertheless, the yeast two-hybrid assay has the potential to provide important information on the specific target(s) of Ptr ToxB action, and could serve to complement the protein-localization studies described above.

In Chapter 2, we showed that Ptr ToxB affected the abundance of many proteins involved in energy metabolism, particularly those related to photosynthesis. The levels of other chloroplast-related proteins were also significantly altered at all time-points. It appears that treatment with Ptr ToxB results in damage to the integrity of the chloroplast membranes, likely through the formation of ROS. Therefore, a follow-up study could involve an examination of the effects of Ptr ToxB specifically on the chloroplast proteome of the toxin-sensitive wheat genotype 'Katepwa', which might provide insights into Ptr ToxB action at the organelle level. Intact chloroplasts could be isolated from toxin-sensitive wheat leaf tissue and incubated with Ptr ToxB under light and with gentle agitation; the chloroplast fractions could be collected at various time-points and the organelle proteome analyzed by 2-DE. Furthermore, specific changes in the chloroplast proteome could be validated by Western blotting, focusing on photosynthesis-related proteins such as thylakoid lumen or membrane proteins and photosystem II-related proteins [including oxygen evolving enhancer protein 1 (OEE) and D1 protein]. The work on the effect of Ptr ToxB on toxin-sensitive wheat tissue, and specifically on the chloroplasts, could allow us to better understand the mechanism(s) by which photosynthesis is inhibited.

Another component of future research could involve the detection of ToxB protein in isolates of *P. tritici-repentis* lacking Ptr ToxB activity or in other ascomycete fungi carrying *ToxB* homologs. In the case of *P. tritici-repentis*, toxin production has usually been examined by Western blotting analysis. However, depending on the titer of the antibodies used and the sensitivity of the assay,

failure to detect Ptr ToxB does not necessarily mean that it is not being produced. As technology has advanced, it is now possible to detect a very low amount of protein at the cell level using real-time PCR and antibodies, with tools such as the TaqMan® Protein Assay (Applied Biosystems). Information on the production of the ToxB protein, both in isolates of *P. tritici-repentis* that do not possess toxin activity and in other ascomycetes, could provide clues as to whether or not the *ToxB* homologs are active genes. All of these studies would build on the work presented here and help to answer questions raised during the current research.

5.5. Concluding remarks

Tan spot remains an important disease of wheat, and its effective management will require an integrated approach. While the research presented in this dissertation is basic in nature, it has the potential to contribute to successful tan spot control. By elucidating the role of effectors such as Ptr ToxB, we can gain insights into the molecular mechanisms behind plant disease development. In addition, the current study will serve to guide work on other toxins or effectors from related fungi. These insights can in turn be used to develop knowledge-based plant protection strategies. For instance, by elucidating the role of Ptr ToxB in the virulence or pathogenicity of *P. tritici-repentis* and its possible contribution to virulence in other fungi such as *P. teres*, we can focus resistance screening efforts against this effector and the other Ptr toxins, as well as gain an appreciation of the potential risks to wheat and other cereal crops. Therefore, an increased understanding of the underlying mechanisms of fungal virulence may be used to

develop crop cultivars and varieties with enhanced disease resistance, as well as contributing fundamental knowledge to plant science.

5.6. References

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Appendix A

A proteomic evaluation of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, reveals major differences between pathogenic and non-pathogenic isolates*

A.1. Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* [Died.] Shoem.) causes tan spot, an economically important foliar disease of wheat with a worldwide distribution. Tan spot is associated with the development of extensive chlorotic and/or necrotic lesions on the leaves of susceptible host genotypes, resulting in a reduction in photosynthetic area. Isolates of *P. tritici-repentis* are classified into races based on their ability to induce these symptoms on a set of wheat differential hosts (Lamari *et al.*, 1995) and, currently, eight races of the pathogen are known. As the differential set is expanded, additional races will likely be identified (Strelkov and Lamari, 2003). The necrosis and chlorosis induced by *P. tritici-repentis* result from the differential production of host-specific toxins (HSTs) by isolates of the fungus. The proteinaceous toxin Ptr ToxA [syn. Ptr toxin, Ptr necrosis toxin, and ToxA (Ciuffetti *et al.*, 1998)] causes necrosis on sensitive wheat genotypes, and is

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encoded by the single copy gene, *ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Another proteinaceous toxin, Ptr ToxB [syn. Ptr chlorosis toxin (Ciuffetti *et al.*, 1998)] (Strelkov *et al.*, 1999), encoded by the multicopy *ToxB* gene (Martinez *et al.*, 2001; Strelkov and Lamari, 2003), induces chlorosis on sensitive wheat lines and cultivars. A third HST, Ptr ToxC, also induces chlorosis, but not on the same host genotypes as Ptr ToxB (Gamba *et al.*, 1998). Unlike the other Ptr toxins, Ptr ToxC is not a protein, but rather a polar, non-ionic, low molecular mass compound (Effertz *et al.*, 2002).

Most research suggests that susceptibility to the races of *P. tritici-repentis* and sensitivity to the toxins produced by those races co-segregate (Gamba and Lamari, 1998; Gamba *et al.*, 1998; Lamari and Bernier, 1991), indicating that the Ptr toxins serve as pathogenicity factors for the fungus, *sensu* Yoder (1980). Races 1, 2, 7 and 8 of *P. tritici-repentis* produce Ptr ToxA, races 5, 6, 7 and 8 produce Ptr ToxB, whilst races 1, 3, 6 and 8 produce Ptr ToxC. In contrast, race 4 isolates do not produce active forms of the toxins and are considered avirulent. Interestingly, in addition to occurring as a multicopy gene in Ptr ToxB-producing isolates of *P. tritici-repentis*, homologs of *ToxB* have also been found in isolates of races 3 and 4 that do not possess detectable Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov and Lamari, 2003; Strelkov *et al.*, 2006). The form of *ToxB* [termed *toxb* by Martinez *et al.* (2004)] found in race 4 isolates shares 86% homology with the gene found in wild-type, virulent isolates Martinez *et al.*, 2004; Strelkov and Lamari, 2003). Amaike *et al.* (2008) examined expression of the *ToxB* homolog from race 4 by quantitative reverse transcriptase (RT)-PCR,

and found that it was transcribed, albeit at very low levels, *in planta* and *in vitro*. Abundance of the transcript was greatest in conidia, but was still several orders of magnitude lower than in conidia of a virulent race 5 isolate (Amaike *et al.*, 2008). Production of the protein encoded by the *ToxB* homolog in race 4 was not examined, although when expressed heterologously, it possessed very little chlorosis-inducing activity (Kim and Strelkov, 2007). Recently, *ToxB* homologs have also been reported in the *P. tritici-repentis* sister species *P. bromi* and other ascomycetes, suggesting that the toxin may have arisen in a common progenitor of the Ascomycota (Andrie *et al.*, 2008).

It was previously suggested that the host-specificity and virulence conferred by the Ptr toxins is superimposed on the general pathogenic ability of *P. tritici-repentis* (Lamari and Gilbert, 1998; Strelkov and Lamari, 2003). This suggestion is supported by the observation that isolates of the fungus can form appressoria and penetrate the epidermal cells of resistant host genotypes, before further growth is stopped in the mesophyll (Lamari and Bernier, 1989a; Larez *et al.*, 1986). Moreover, transformation of an isolate of *P. tritici-repentis* with Ptr ToxA was a sufficient condition for virulence on a Ptr ToxA-sensitive genotype of wheat (Ciuffetti *et al.*, 1997). However, most studies have focused on the interaction between toxin-insensitive (resistant) hosts and toxin-producing (virulent) fungal isolates. Amaike *et al.* (2008) compared the infection process of a truly avirulent isolate (i.e., producing no active toxins) of race 4 with that of a highly virulent race 5 isolate (producing Ptr ToxB). They found that while there was no difference between the isolates in terms of the conidial germination rates

and the number of germ tubes formed per conidium, the number of appressoria produced and the speed of their formation were significantly lower in the avirulent isolate. In addition, a weakly virulent isolate (producing low amounts of active Ptr ToxB) developed intermediate numbers of appressoria (Amaike *et al.*, 2008).

The fact that the number of appressoria formed by virulent, weakly virulent and avirulent isolates was strongly correlated with *ToxB* gene expression led Amaike *et al.* (2008) to suggest that the toxin could have other roles in pre-penetration processes, or that additional factors could contribute to the reduction in general pathogenic ability of avirulent isolates. In the current study, we examined the production of Ptr ToxB by isolates representing the virulent race 5 and avirulent race 4 of *P. tritici-repentis*, and compared the secretomes and mycelial proteomes of these races. Information on whether or not race 4 isolates produce Ptr ToxB in detectable amounts is important for understanding the role, if any, of the *ToxB* homolog found in this race. Moreover, comparison of the complete proteomes of virulent and avirulent races could help identify differences that explain a reduced underlying pathogenic ability in race 4, irrespective of toxin production. To our knowledge, this is the first proteomic analysis of *P. tritici-repentis*.

A.2. Materials and methods

A.2.1. Fungal material

Isolates 90-2 and Alg3-24, representing races 4 and 5, respectively, of *P. tritici-repentis*, were selected for use in this study. Race 4 isolate 90-2 was

collected in Manitoba, Canada, and is avirulent (Lamari *et al.*, 1998), lacking any detectable toxin activity. Race 5 isolate Alg3-24 was obtained from eastern Algeria and is virulent (Lamari *et al.*, 1995), producing active Ptr ToxB (Strelkov *et al.*, 1999). The isolates were grown in darkness at room temperature on V8-potato dextrose agar medium (Lamari and Bernier, 1989a) until the colonies reached approximately 5 cm in diameter. In order to induce sporulation, mycelia were flattened using the bottom of a sterile glass test tube, incubated under light at room temperature for 18 h, and then placed in darkness at 15^{o}C for 24 h. The colonies were flooded with sterile distilled water (sd-H_2O) and the conidia dislodged by gentle scraping with a sterilized loop (Lamari and Bernier, 1989b). The resulting conidial suspensions were filtered through two layers of sterilized cheesecloth, quantified using a hemocytometer, and adjusted to a final concentration of 3500 conidia/mL with sd-H_2O. A 1 mL volume of conidial suspension was used to inoculate 100 mL of Fries medium (Dhingra and Sinclair, 1986) in a 200 mL Erlenmeyer flask, with the remainder used to inoculate wheat plants and produce spore germination fluids, as described below. Liquid cultures were incubated in darkness at room temperature for 10 or 20 days, and the mycelial mats were separated from the culture medium by centrifugation at 13800 × *g* for 20 minutes. Both the mycelium and culture fluids were retained for protein extraction.

A.2.2. Plant material and inoculation

Plants of wheat line 6B662 (sensitive to Ptr ToxB, resistant to race 4 and susceptible to race 5) and cv. Erik (insensitive to Ptr ToxB and resistant to both

races) were grown in 12.7 cm diameter plastic pots filled with Metro Mix® 220 soil (Grace Horticultural Products, Ajax, ON) at a density of five seedlings per pot, with watering and fertilization as required. Seedlings were maintained in a growth cabinet with a 16 h photoperiod (180 μmol/m^2/s) at 20°C/18°C until inoculation at the 2-3 leaf stage. The seedlings were inoculated with a suspension of 3500 conidia/mL, to which 10 drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per L were added, as described by Lamari and Bernier (1989a). Each host genotype was inoculated with each isolate and leaves were examined for symptom development one week after inoculation.

A.2.3. Spore germination fluids

Conidial suspensions (40 mL; 3500 conidia/mL) were placed in 14 cm diameter sterile Petri dishes containing 7 surface-sterilized leaf segments (10 cm long) of wheat line 6B662 or cv. Erik. The Petri dishes were incubated for 48 h in darkness at room temperature, and the spore germination fluids harvested by decanting the suspension into 50 mL centrifuge tubes. Cell-free spore germination fluids were obtained by centrifugation at 9500 × *g*. The resulting supernatants were lyophilized, re-suspended in 200 μL sd-H_2O, and analyzed directly by Western blotting as described below.

A.2.4. Protein extraction and processing

The liquid medium collected from 10 or 20-day-old cultures of the fungus was filtered through a 0.22 μm cellulose nitrate filter (Micron Separation Inc., Westboro, Mass.) and dialyzed (molecular mass cut-off: 1000 Da) against sd-H_2O at 4°C. Dialyzed samples were lyophilized to concentrate the secreted proteome

and re-suspended in 1.2 mL rehydration/sample buffer (Bio-Rad, Mississauga, ON) containing 2 mM tributylphosphine (Bio-Rad). Mycelium was washed twice by centrifugation (13800 × *g*, 10 minutes, 4^{o}C) with 10 mL of 10 mM Tris-HCl containing complete (EDTA-free) protease inhibitor (Roche, Indianapolis, IN, USA), pH 7.8. The washed mycelia were lyophilized, ground to a fine powder in liquid nitrogen, and the proteins extracted for 2-DE as described by Yajima and Kav (2006). Prior to 2-DE, samples were desalted using a ReadyPrepTM 2-D Cleanup Kit (Bio-Rad), according to the manufacturer's instructions, and the protein concentrations determined using a modified Bradford (1976) assay with BSA (Pierce Biotechnology Inc., Rockford, IL) as a standard. Three separate protein extracts were prepared from each of three independently grown cultures of each isolate, for analysis of the secretome and mycelial proteome. Both 10 and 20-day-old fungal cultures were analyzed by Western blotting, while 10-day-old cultures were used in the analysis of the secretome and mycelial proteome. Spore germination fluids were examined only by Western blotting, because of very low total protein concentrations; the spore germination fluids were concentrated but not processed further prior to analysis.

A.2.5. Two-dimensional gel electrophoresis

For analysis of the secretome, 7-cm IPG strips (pH 4-7, linear, Bio-Rad) were rehydrated overnight at room temperature with 50 μg protein in 125 μL rehydration/sample buffer containing 2 mM tributylphosphine. To analyze the mycelial proteins, 17-cm IPG strips (pH 3-10, linear, Bio-Rad) were rehydrated with 500 μg protein in 300 μL of the same buffer. Less protein was used in the

secretome versus mycelial analysis because of the considerably lower amounts of protein secreted into the medium. Isoelectric focusing to separate proteins based on p*I* was conducted on a PROTEAN IEF cell (Bio-Rad) as previously described (Cao *et al.*, 2008). After equilibration of the focused proteins, the IPG strips were embedded in 0.5% agarose (Bio-Rad), placed on top of 1 mm 13% SDS-polyacrylamide gels, and separated in the second-dimension using the Mini PROTEAN 3 system or a PROTEAN II xi cell (Bio-Rad), at a constant voltage of 150 or 90 V, respectively. Gels were stained using a Colloidal Blue Staining Kit (Invitrogen Co., Carlsbad, CA), destained in sd-H_2O, and scanned with a GS-800 Calibrated Densitometer (Bio-Rad).

A.2.6. Gel analysis and protein identification

All 2-DE gel images were analyzed using PDQuest software (Version 7.3.1, Bio-Rad), with a set of 12 images examined for each of the mycelial and secretome analyses, consisting of two gels from each of three independent replications with Alg3-24 and 90-2. The automated detection and matching tools of the PDQuest software were used to analyze the images, with occasional manual elimination of artifacts and matching reinforcement. The intensities of the matched protein spots were determined using the spot quantification tool and compared between Alg3-24 and 90-2 using the Student's t-test feature of the software. The intensities of spots showing significant differences ($p < 0.05$) between the isolates were exported to Microsoft Excel (Microsoft Co., Redmond, WA) and the fold changes calculated. In the analysis of the secretome, all spots showing significant fold changes were excised with a sterile scalpel, and the

protein identities determined by ESI-quadrupole-TOF MS/MS (ESI-q-TOF MS/MS), as described by Cao *et al.* (2008), at the Institute for Biomolecular Design, University of Alberta, Edmonton, Canada. In the analysis of the mycelial proteome, only those spots showing significant changes greater than three-fold were excised for protein identification by ESI-q-TOF MS/MS. The raw data obtained by MS/MS were converted to MASCOT generic files and used to search the National Center for Biotechnology Information (NCBI) non-redundant database using the MASCOT online search engine (www.matrixscience.com). Search parameters included fixed modification of carbamidomethyl, 1 missed trypsin cleavage, peptide tolerance of ± 0.6 Da, peptide charge of 2+ or 3+, and peptide mass tolerance of 20 ppm. Identified proteins were classified into functional categories based on the groupings used by the Munich Information Center for Protein Sequences (MIPS) (Ruepp *et al.*, 2004).

A.2.7. Western blotting

Polyclonal antibodies were raised against hexahistidine (His)-tagged Ptr ToxB from Alg3-24, which had been heterologously expressed in *Escherichia coli* and purified by affinity chromatography as described by Kim and Strelkov (2007). The antibodies were raised in rabbits at the Biosciences Animal Service Unit, University of Alberta, and stored as a stock preparation at -20^{o}C for Western blotting analysis. Total soluble proteins from concentrated spore germination fluids, mycelium and culture medium of 10 or 20-day-old liquid cultures of *P. tritici-repentis* were subjected to SDS-PAGE using a tris-tricine buffer system

(Schägger and von Jagow, 1987), and transferred to PVDF membranes (Bio-Rad) according to the protocols of Bjerrum and Schafer-Nielsen (1986).

After transfer, the membranes were incubated overnight at 4°C, with agitation, in a blocking buffer consisting of 5% (w/v) non-fat dry milk in TBS (50 mM Tris, 150 mM NaCl, pH 7.5). They were then rinsed three times (5 min per rinse) with TTBS (0.05% [v/v] Tween 20 [polyoxyethylene sorbitol monolaurate] in TBS), and incubated for 1 h with the polyclonal antibodies against His-tagged Ptr ToxB, diluted 1/3000 in antibody buffer (1% [w/v] non-fat dry milk in TTBS). The membranes were washed three times in TTBS, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), diluted 1/3000 in antibody buffer. The membranes were washed three more times in TTBS, once in TBS, and stained using a tetramethyl benzidine substrate kit for peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) as per the manufacturer's instructions. Blot images were recorded with a GS-800 Calibrated Densitometer (Bio-Rad).

A.3. Results and discussion

A.3.1. Symptom development

One week after inoculation with race 5 isolate Alg3-24, extensive chlorosis was visible on the leaves of the Ptr ToxB-sensitive wheat line 6B662 (Fig. A-1). In contrast, race 4 isolate 90-2 induced the development of small chlorotic flecks. Neither isolate caused chlorosis on cv. Erik, although small, localized necrotic lesions typical of a resistant reaction (Lamari and Bernier,

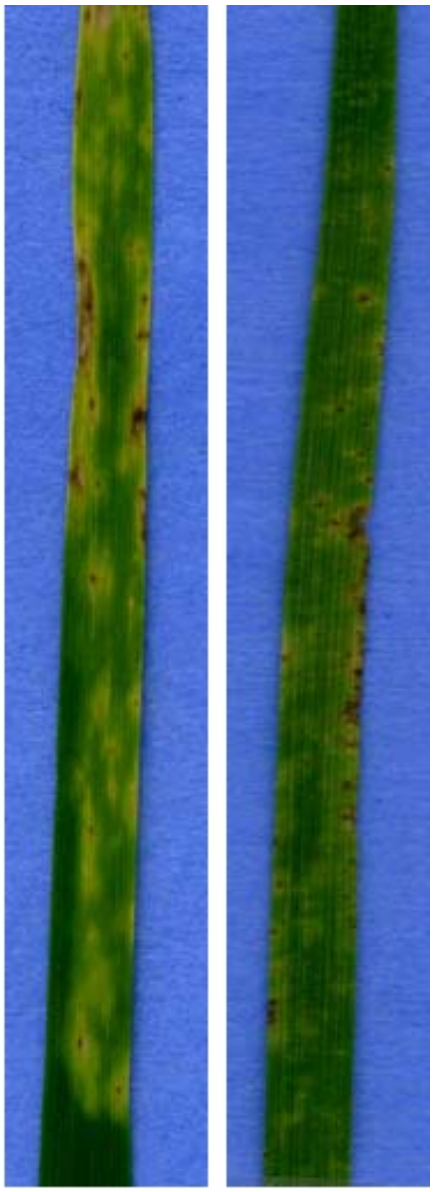


Figure A-1. Symptoms induced by race 5 isolate Alg3-24 and race 4 isolate 90-2 of *Pyrenophora tritici-repentis* on wheat line 6B662. This line is sensitive to Ptr ToxB, susceptible to race 5 and resistant to race 4. Leaves are shown 7 days after inoculation. Neither isolate caused chlorosis on wheat cv. Erik, which is insensitive to the toxin (not shown).

1991) were observed. The host reactions confirmed the virulence designations of both isolates.

A.3.2. Occurrence of Ptr ToxB

Polyclonal antibodies raised against His-tagged Ptr ToxB reacted with a protein approximately 6 kDa in size, corresponding to Ptr ToxB (Kim and Strelkov, 2007), in spore germination fluids and 10 and 20-day-old culture fluids of race 5 isolate Alg3-24 (Fig. A-2). The intensity of the band was stronger in 20-day-old versus 10-day-old culture fluids (Fig. A-2A), indicating the presence of greater quantities of toxin at 20 days. However, the strongest band was observed in the spore germination fluids (Fig. A-2B), which supports the role of Ptr ToxB as a pathogenicity factor required at the earliest stages of infection. The intensity of the toxin band produced in the spore germination fluids was similar regardless of the leaf genotype incubated with the spores (data not shown). This is consistent with the constitutive expression of the *ToxB* gene observed in liquid culture and its expression in both resistant and susceptible host genotypes after inoculation (Amaike *et al.*, 2008). A protein band also reacted with the Ptr ToxB antibodies in 20-day-old mycelium of Alg3-24, likely reflecting the presence of the toxin in the hyphae prior to its secretion into the growth medium.

No band was observed in mycelium, spore germination or culture fluids of race 4 isolate 90-2 (Fig. A-2), indicating that it does not produce Ptr ToxB in detectable amounts. Thus, although the *ToxB* homolog is transcribed at low levels in conidia and mycelia of race 4 (Amaike *et al.*, 2008), this does not result in accumulation of a protein that is detectable using our antibodies. His-tagged Ptr

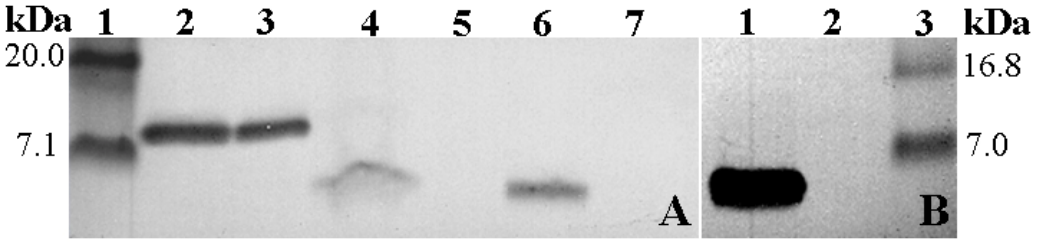


Figure A-2. Western blot analysis of protein from race 5 isolate Alg3-24 and race 4 isolate 90-2 of *Pyrenophora tritici-repentis* with polyclonal antibodies specific to Ptr ToxB. Panel A: Prestained SDS-PAGE Standards, Broad Range (10 µg; Bio-Rad, Mississauga, ON) (lane 1), heterologously expressed His-tagged Ptr ToxB from Alg3-24 (lane 2), heterologously expressed His-tagged Ptr ToxB from 90-2 (lane 3), concentrated culture fluids (10 µg total protein) from 10-day-old cultures of Alg3-24 (lane 4) and 90-2 (lane 5), and concentrated culture fluids (1 µg total protein) from 20-day-old cultures of Alg3-24 (lane 6) and 90-2 (lane 7). Panel B: spore germination fluids (1 µg total protein) from Alg3-24 (lane 1) and 90-2 (lane 2), and Kaleidoscope Prestained Standards (10 µg; Bio-Rad).

ToxB from isolates 90-2 and Alg3-24 was included in the assay and also reacted with the antibodies (Fig. A-2A); the larger mass of the heterologously expressed Ptr ToxB resulted from the presence of a portion of the signal peptide in these proteins (Kim and Strelkov, 2007). Inclusion of these controls demonstrated that, although raised against heterologously expressed Ptr ToxB from Alg3-24, the antibodies also reacted strongly with the form of the toxin from 90-2.

A.3.3. Secretomes of races 4 and 5

A.3.3.1. Differentially expressed secreted proteins

Analysis of the corresponding sets of 2-DE gels revealed an average (± 1 S.E.) of 67 ± 0 protein spots in the secretomes of race 4 isolate 90-2 and race 5 isolate Alg3-24, of which 29 differed in intensity between the isolates. Identities for nine of these proteins could be obtained by MS/MS (Fig. A-3; Table A-1). It is possible that the inability to identify the others resulted from an insufficient amount of protein in those spots, and/or that those proteins are not found in the protein databases used to search for similarities to the mass spectra generated in the analysis (Yajima and Kav, 2006). Highly significant ($p < 0.0001$) correlations were found between the observed and predicted molecular masses and p*I* values of differentially expressed secreted (Table A-1) and mycelial (Table A-2) proteins, suggesting experimental consistency. Nonetheless, for a few proteins, the observed and predicted values were different, and the spots may have represented degradation products of larger proteins. The intensity of all identified secreted proteins was greater in Alg3-24 than in 90-2, with the exception of spot 3 (Fig. A-

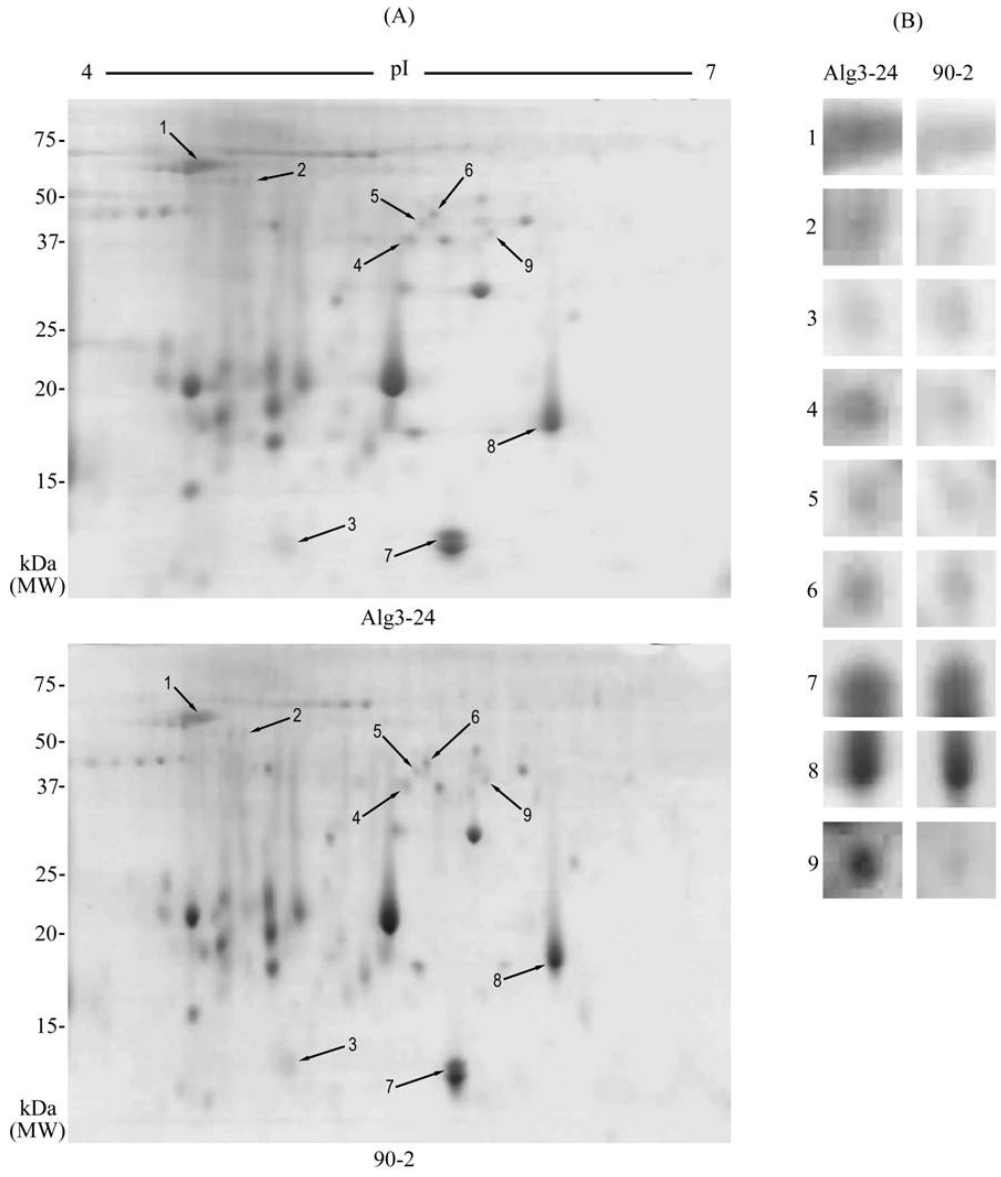


Figure A-3. Panel A: Representative 2-D gels of secreted proteins in the 10-day-old culture medium of race 5 isolate Alg3-24 and race 4 isolate 90-2 of *Pyrenophora tritici-repentis*. The gels were stained with colloidal blue. Spots showing significant differences ($p < 0.05$) in intensity between the isolates and identified by MS/MS are indicated by arrows and numbers; these proteins are listed in Table A-1. Panel B: A closer view of differentially abundant proteins identified by MS/MS.

Table A-1. Identification of proteins found to be differentially abundant in the secretomes of non-pathogenic isolate 90-2 and pathogenic isolate Alg3-24 of *Pyrenophora tritici-repentis*.

Spot	Protein identity[a)]	MS/MS		Access ID[b)]	Predicted MW(Da)/p*I*	Observed MW(Da)/p*I*	Fold change[c)] ± SE
		PM[d)]/Seq cov./Score[e)]	Sequence (charge)				
1	Alpha-mannosidase [*Aspergillus saitoi*]	2/2%/55 (>33)	R.YLGGMLSGYDLLK.G (2+) R.YLGGMLSGYDLLK.G (2+)	gi|1171477	56011/4.92	66667/4.79	4.0 ± 0.9
2	Hypothetical protein SNOG_06620 [*Phaeosphaeria nodorum* SN15]	1/1%/40 (>32)	K.LVVEYQTDKR.L (2+)	gi|160707158	102751/5.41	55952/4.98	37.9 ± 22.3
3	Hypothetical protein CIMG_05331 [*Coccidioides immitis* RS]	1/32%/48 (>32)	K.LSAEAPILFMFKK.N (2+)	gi|119190567	4726/9.30	13770/5.10	-1.6 ± 0.3
4	Hypothetical protein SNOG_00606 [*Phaeosphaeria nodorum* SN15]	2/6%/64 (>32)	K.LGEALGWAQEAGLK.V (2+) R.FIEAQLDAYEK.A (2+)	gi|111070981	44864/5.28	37325/5.43	7.5 ± 2.3
5	Chitinase [*Rhizopus microsporus* var. *oligosporus*]	1/2%/44 (>34)	K.LVVGMPLYGR.G (2+)	gi|1565203	46104/6.35	41875/5.55	3.2 ± 0.7
	Unnamed protein product [*Aspergillus oryzae*]	1/2%/44 (>34)	K.IVVGMPIYGR.A (2+)	gi|83767689	50343/5.65		
6	Chitinase [*Rhizopus microsporus* var. *oligosporus*]	5/2%/47 (>33)	K.LVVGMPLYGR.G (2+)	gi|1565203	46104/6.35	44042/5.60	5.2 ± 1.7
	Unnamed protein product [*Aspergillus oryzae*]	5/2%/47 (>33)	K.IVVGMPIYGR.A (2+)	gi|83767689	50343/5.65		
	tRNA 2'-O-methyltransferase Trm13 (predicted) [*Schizosaccharomyces pombe* 972h-]	1/2%/35 (>33)	R.WRTLSTFAR.E (2+)	gi|19114826	47762/9.31		
7	AER306Cp [*Ashbya gossypii ATCC* 10895]	1/3%/36 (>35)	K.GQMGNVDDIQAKLDK.R (2+)	gi|45190908	47613/8.33	13689/5.67	1.1 ± 0.1
8	Hypothetical protein AN0203.2 [*Aspergillus nidulans* FGSC A4]	24/3%/39 (>36)	K.TGMVMAVQLTR.T (2+)	gi|67515843	32536/6.20	18333/6.01	12.3 ± 2.9

9	Exo-1,3-beta-glucanase [*Cochliobolus carbonum*]	1/2%/34 (>30)	R.AVGFNWGSEK.I (2+)	gi|7638024	47390/6.14	38083/5.79	6.7 ± 2.5

a) Proteins were identified using ESI-quadrupole-TOF MS/MS data.
b) Access ID is the access identification of the protein from the non-redundant database search.
c) Fold-change refers to the mean difference in intensity of the matched protein spots in Alg3-24 relative to 90-2.
d) Number of peptides matched.
e) Ion score is −10 log (P), where P is probability that the observed match is a random event; individual ion scores larger than the threshold values in parentheses indicates identity or extensive homology ($p < 0.05$).

3; Table A-1), which was greater in 90-2. Secreted proteins belonged to various functional categories, including general metabolism (3 spots), cell rescue, defense and virulence (1 spot), cell cycle and DNA processing (1 spot), and unclassified proteins (4 spots) that have not yet been functionally characterized.

A.3.3.2. Metabolism

The intensity of α-mannosidase (spot 1) was 4.0-fold higher in Alg3-24 than in 90-2 (Fig. A-3; Table A-1). This enzyme catalyzes the specific cleavage of α-mannose residues from protein-linked high mannose oligosaccharides, and was previously found to be secreted by *Aspergillus oryzae* (Akao *et al.*, 2006). The secretion of α-mannosidase by *P. tritici-repentis* may be related to the degradation of the host cell wall, since mannose has been found in small amounts in the cell wall fraction of wheat (Obel *et al.*, 2002). Studies with an α-mannosidase mutant lacking non-specific activity revealed that the enzyme does not appear to be a vital component of the vegetative cell cycle in *Saccharomyces cerevisiae* (Cueva *et al.*, 1990), suggesting that the lower level of expression of α-mannosidase in isolate 90-2 of *P. tritici-repentis* does not affect its basic biological fitness.

Two spots identified as chitinases (spots 5, 6) were 3.2- and 5.2-fold more abundant in the secretome of Alg3-24 versus that of 90-2 (Fig. A-3; Table A-1). Chitinases hydrolyze chitin, which serves as a structural polysaccharide in fungi and other organisms (Sahai and Manocha, 1993). Fungal chitinases are localized in the periplasmic space and the plasma membrane, and are thought to have autolytic, nutritional, and morphogenetic roles. Chitinases also have roles in the early events of host-microbe interactions involving biotrophic and

necrotrophic mycoparasites, entomopathogenic fungi and vesicular arbuscular mycorrhizal fungi (Sahai and Manocha, 1993), and have been classified as cell rescue/defense proteins in some studies (Bernardo *et al.*, 2007). Given that chitin is not a component of wheat (Obel *et al.*, 2002), it is possible that chitinase synthesis in *P. tritici-repentis* was induced by chitin in the yeast extract of the Fries medium (Dhingra and Sinclair, 1986); synthesis of chitinase can be induced on chitin-containing medium and repressed by glucose and N-acetylglucosamine (Ulhoa and Peberdy, 1991). Although a direct role in the pathogenicity of *P. tritici-repentis* is not clear, the greater abundance of chitinases in the secretome of Alg3-24 relative to that of 90-2 suggests that the former can better utilize semi-synthetic media such as Fries. In addition, the involvement of chitinases in cell wall morphogenesis (Sahai and Manocha, 1993) could indicate more vigorous growth and/or greater plasticity of the cell walls in Alg3-24.

A.3.3.3. Cell rescue, defense and virulence

Expression of exo-1,3-β-glucanase (spot 9) was 6.7-fold greater in the secretome of the virulent versus avirulent isolate (Fig. A-3; Table A-1). This enzyme sequentially releases glucose residues from the non-reducing terminus of a β-glucan chain (Martin *et al.*, 2007). β-Glucans are polymers consisting of glucose residues joined in a β-configuration by glycosidic bonding, and are widely present in plants as a component of the cell walls (Stone and Clarke, 1992). In addition to their role in the degradation of exogenous β-glucans, fungal β-glucanases are thought to play important roles in morphogenesis, mobilization of β-glucans in response to carbon and energy metabolism, cell wall growth and

extension, autolysis and starvation (Martin *et al.*, 2007). Recently, a study on moldy-core disease of apples demonstrated that the virulence of isolates of *Alternaria alternata* was correlated with their capacity to produce glucanase (Reuveni *et al.*, 2007). Thus, the higher expression of β-glucanase in isolate Alg3-24 of *P. tritici-repentis* may reflect a greater general pathogenic ability in this isolate relative to 90-2, since this would result in an increased capacity to degrade host cell walls, facilitating infection and uptake of host nutrients.

A.3.3.4. Other secreted proteins

The remaining differentially expressed spots in the secretomes of Alg3-24 and 90-2 were mostly hypothetical proteins (spots 2, 3, 4, 8), although there was also a spot (no. 7) matching a recombination DNA repair protein (Fig. A-3; Table A-1). Ptr ToxB was not detected by 2-DE in the secretomes of either Alg3-24 or 90-2, despite its occurrence in the former as determined by Western blotting (Fig. A-2A). The small mass of Ptr ToxB likely precluded its detection by 2-DE under the conditions used. Moreover, the amount of Ptr ToxB present at 10 days in culture fluids of Alg3-24 was very low (Fig. A-2A).

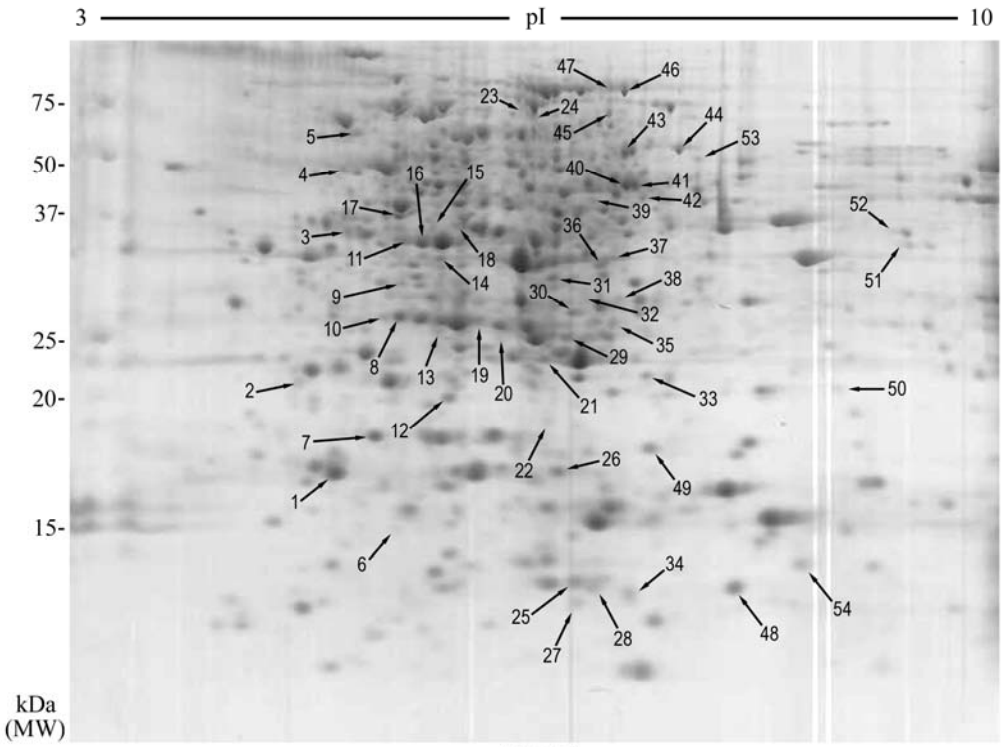
A.3.4. Mycelial proteomes of races 4 and 5

A.3.4.1. Differentially expressed mycelial proteins

An average of 565 ± 0 protein spots were identified in the mycelial proteomes of race 4 isolate 90-2 and race 5 isolate Alg3-24 of *P. tritici-repentis*. A total of 104 of these spots showed differences in intensity greater than 3-fold between the isolates, of which 54 were identified by MS/MS (Fig. A-4; Table A-2). Of those, 40 proteins were more abundant in Alg3-24, and 12 were more

Figure A-4. Panel A: Representative 2-D gels of mycelial proteins in 10-day-old cultures of race 5 isolate Alg3-24 and race 4 isolate 90-2 of *Pyrenophora tritici-repentis*. The gels were stained with colloidal blue. Spots showing significant differences ($p < 0.05$) in intensity (>3-fold) between the isolates and identified by MS/MS are indicated by arrows and numbers; these proteins are listed in Table A-2. Panel B: A closer view of differentially abundant proteins identified by MS/MS.

(A)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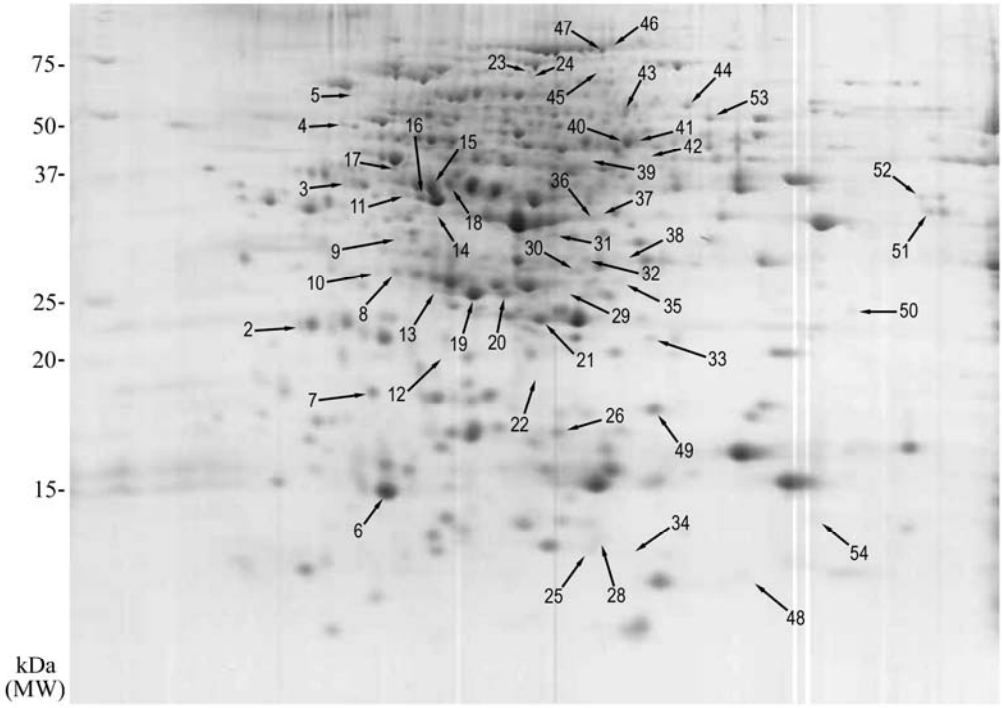
Alg3-24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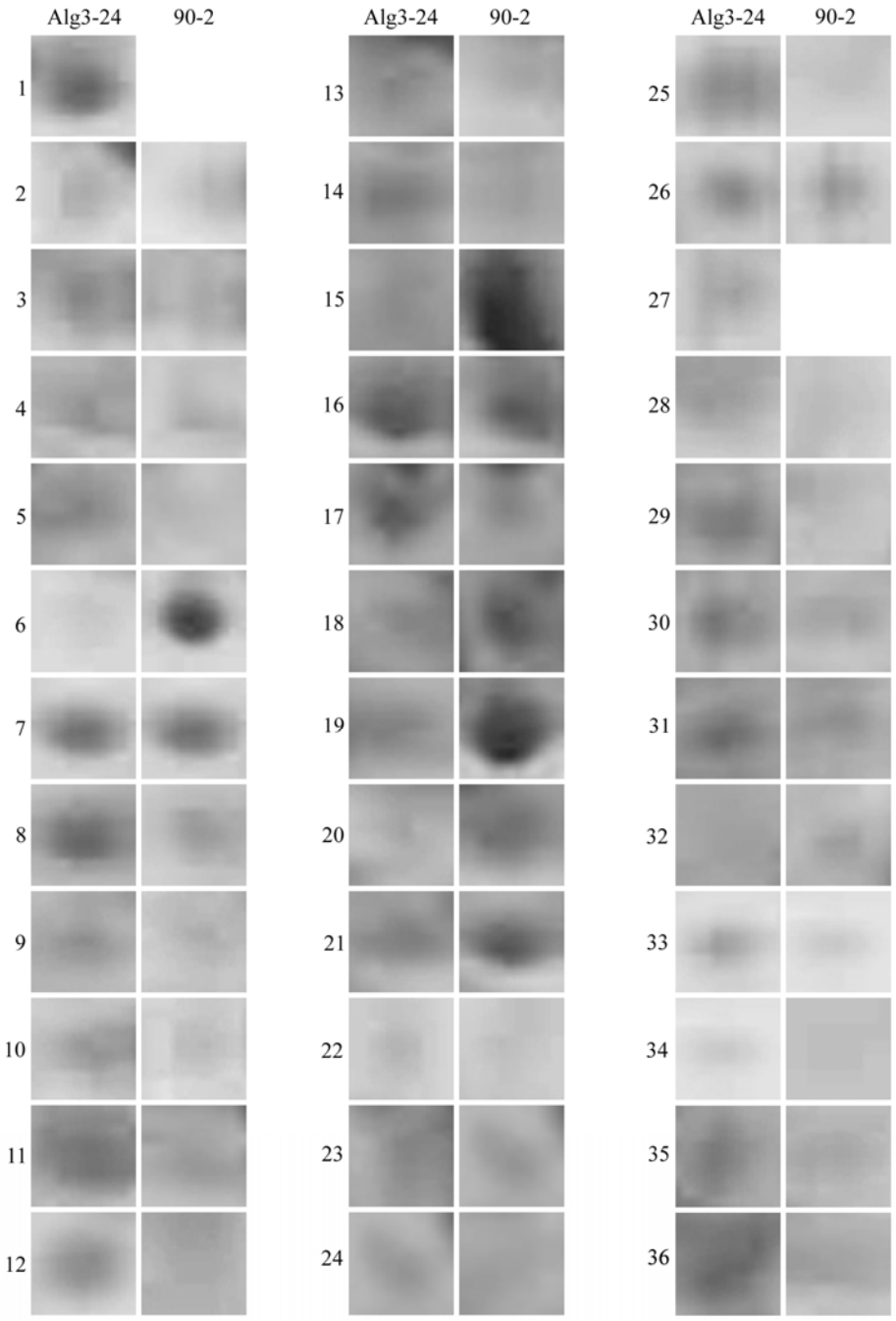
90-2

(B)


Alg3-24
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Alg3-24
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Alg3-24
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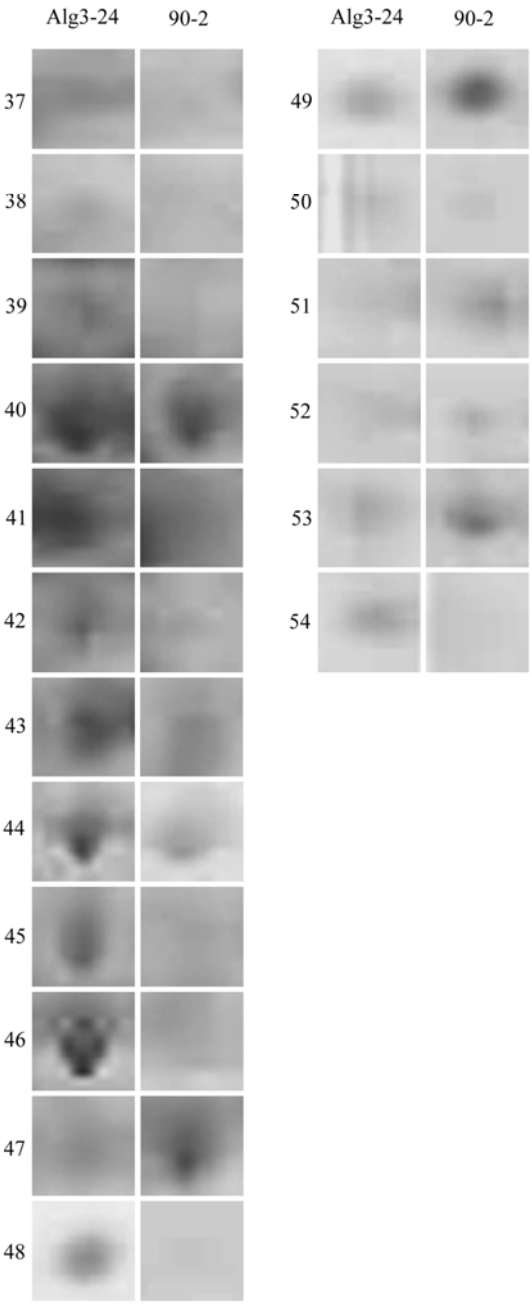
(B)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Alg3-24
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Table A-2. Identification of proteins found to be differentially abundant (> 3-fold) in the mycelial proteomes of non-pathogenic isolate 90-2 and pathogenic isolate Alg3-24 of *Pyrenophora tritici-repentis*.

Spot	Protein identity[a)]	MS/MS		Access ID[b)]	Predicted MW(Da)/p*I*	Observed MW(Da)/p*I*	Fold change ± SE[c)]
		PM[d)]/Seq cov./Score[e)]	Sequence (charge)				
1	Hypothetical protein SNOG_11745 [*Phaeosphaeria nodorum* SN15]	4/28%/135 (>33)	K.GQMSVLEALK.G (2+) K.LALIHDGLAR.G (2+) K.LVVALCSEHKIPLIK.V (2+) K.QLGEWAGLCQIDR.E (2+)	gi|160703345	18619/5.15	17069/5.04	Unique to Alg3-24
	Hypothetical protein BC1G_05503 [*Botryotinia fuckeliana* B05.10]	8/14%/103 (>33)	K.ELLAGFAAGEIDK.L (2+) K.ELLAGFAAGEIDKLAETK.G (2+)	gi|154313003	14494/5.05		
2	Heat shock 70 kd protein cognate 1 [*Magnaporthe grisea* 70-15]	2/4%/48 (>30)	K.FELTGIPPAPR.G (2+) K.ELEAVANPIMMK.F (2+)	gi|145605667	57091/4.95	21923/4.77	8.7 ± 3.4
3	Hypothetical protein FG01241.1 [*Gibberella zeae* PH-1]	4/8%/53 (>33)	K.LGGELTVDER.N (2+) R.DSTLIMQLLR.D (2+)	gi|46108718	27620/4.94	36077/5.13	5.2 ± 1.4
	Hypothetical protein MGG_13806 [*Magnaporthe grisea* 70-15]	4/7%/53 (>33)	K.LGGELTVDER.N (2+) R.DSTLIMQLLR.D (2+)	gi|145603031	30936/4.96		
4	ER chaperone BiP [*Aspergillus oryzae*]	3/3%/120 (>31)	K.TLKPVEQVLK.D (2+) K.KSEVDDIVLVGGSTR.I (2+) K.SEVDDIVLVGGSTR.I (2+)	gi|5597020	73462/4.82	49071/5.08	3.0 ± 0.8
5	ER chaperone BiP [*Aspergillus oryzae*]	2/3%/104 (>31)	K.TLKPVEQVLK.D (2+) K.SEVDDIVLVGGSTR.I (2+)	gi|5597020	73462/4.82	61538/5.19	16.0 ± 10.0
	Hypothetical protein CHGG_09206 [*Chaetomium globosum* CBS 148.51]	2/3%/104 (>31)	K.TLKPVEQVLK.D (2+) K.SEVDDIVLVGGSTR.I (2+)	gi|116202643	72278/4.89		
6	Hypothetical protein SNOG_12251 [Phaeosphaeria nodorum SN15]	5/4%/79 (>32)	K.ALAPEYETAATTLK.E (2+) R.KADSLISYMTK.Q (2+)	gi|111059543	60047/4.66	14786/5.44	-167.4 ± 47.4

7	Translation elongation factor [*Gigaspora margarita*]	2/13/104 (>30)	R.LPLQDVYK.I (2+) K.IGGIGTVPVGR.V (2+)	gi|37653269	16127/6.28	17680/5.31	5.2 ± 1.3
	Elongation factor-1 alpha [*Leucoagaricus* sp. G57]	2/10%/106 (>30)	R.LPLQDVYK.I (2+) K.IGGIGTVPVGR.V (2+)	gi|110349571	19684/5.96		
8	Unnamed protein product [*Candida glabrata*]	2/4%/45 (>35)	K.TFTPEEISSMVLGK.M (2+) K.DAGTIAGLNVLR.I (2+)	gi|50288155	69920/5.06	27769/5.50	5.7 ± 0.6
9	Hypothetical protein SNOG_10727 [*Phaeosphaeria nodorum* SN15]	5/9%/60 (>34)	K.EAVLCDIDEAVIR.L (2+) K.EIHEAAFVLPTFAR.K (3+) K.EIHEAAFVLPTFAR.K (2+)	gi|160702895	33381/5.21	31231/5.50	3.3 ± 0.7
10	Hypothetical protein SNOG_07609 [*Phaeosphaeria nodorum* SN15]	2/3%/44 (>33)	R.LVEVAGLPIR.V (2+)	gi|111063955	29766/5.44	27769/5.38	7.5 ± 1.3
11	Hypothetical protein SNOG_04428 [*Phaeosphaeria nodorum* SN15]	2/3%/55 (>34)	K.IMVINVNDPLAPK.L (2+)	gi|160706273	41471/8.15	35385/5.77	4.9 ± 0.9
12	Hypothetical protein SNOG_08496 [*Phaeosphaeria nodorum* SN15]	11/12%/47 (>33)	K.VQHEILAGLK.Y (2+) K.KFEPETAPSGMLGR.G (3+)	gi|111062544	22479/5.68	20000/5.83	226.4 ± 94.1
13	Hypothetical protein SNOG_07991 [*Phaeosphaeria nodorum* SN15]	3/8%/155 (>30)	R.SAALLESYGIGVPK.G (2+) R.EFYLAILMDR.G (2+) K.IFSIDDLQAAAEK.S (2+)	gi|111063147	47727/5.6	26154/5.75	5.9 ± 3.1
14	Hypothetical protein SNOG_08496 [*Phaeosphaeria nodorum* SN15]	30/20%/116 (>33)	K.LDQNDESLQK.W (2+) K.VQHEILAGLK.Y (2+) K.KFEPETAPSGMLGR.G (3+) K.WSFDIK.K (1+)	gi|111062544	22479/5.68	33631/5.79	4.0 ± 1.1
15	Hypothetical protein SNOG_04428 [*Phaeosphaeria nodorum* SN15]	2/7%/43 (>34)	K.VLGVMALLDEGETDWK.I (2+) K.IMVINVNDPLAPK.L (2+)	gi|160706273	41471/8.15	35033/5.78	- 7.8 ± 2.1
16	Hypothetical protein SNOG_04428 [*Phaeosphaeria nodorum* SN15]	2/5%/108 (>34)	K.IMVINVNDPLAPK.L (2+) K.LNDVEDVER.H (2+)	gi|160706273	41471/8.15	35154/5.67	4.6 ± 0.5
17	Hypothetical protein SNOG_02070 [*Phaeosphaeria nodorum* SN15]	17/8%/111 (>34)	K.TEYLYDEK.T (2+) R.CGVVITGHNR.S (2+) R.SLCTDLAAANNYK.V (2+)	gi|111069162	37864/5.20	39786/5.48	4.0 ± 1.4

18	Transaldolase 1 [*Cochliobolus heterostrophus*]	5/14%/210 (>35)	K.EILQIVPGK.V (2+) K.LASTWEGIK.A (2+) K.SVQQIFNYYK.K (2+) K.TIVMGASFR.S (2+) K.FAADAVTLK.D (2+)	gi|163931305	36110/5.30	35426/5.90	- 6.0 ± 2.2
19	Hypothetical protein SNOG_03889 [*Phaeosphaeria nodorum* SN15]	43/12%/110 (>35)	R.FTQEFAPIFR.L (2+) R.LFDEYDR.Q (2+) R.SVGEFHR.S (2+)	gi|111067974	22471/5.67	24375/6.07	-1781.0 ± 1162.2
	HS30_EMENI 30 KD Heat shock protein [*Aspergillus nidulans* FGSC A4]	33/7%/76 (>35)	K.ASLKDGILSLVVPK.A (3+) K.ASLKDGILSLVVPK.A (2+) K.DGILSLVVPK.A (2+)	gi|67524145	20272/6.10		
	Heat shock protein 30 [*Metarhizium anisopliae*]	29/4%/40 (>35)	K.DGILSIVVPK.A (2+)	gi|88766399	25226/5.54		
20	Hypothetical protein SNOG_03889 [*Phaeosphaeria nodorum* SN15]	9/5%/51 (>34)	R.FTQEFAPIFR.L (2+)	gi|111067974	22471/5.67	24375/6.27	- 12.9 ± 3.8
21	Hypothetical protein SNOG_03572 [*Phaeosphaeria nodorum* SN15]	2/4%/41 (>34)	K.DFTLIASSK.A (2+)	gi|160705926	22761/5.96	22031/6.55	-3.7 ± 0.3
22	Amidase family protein, putative [*Aspergillus fumigatus* Af293]	3/1%/43 (>33)	K.ADAFVVTK.L (2+)	gi|70996580	55292/5.66	19052/6.65	32.4 ± 10.9
	Hypothetical protein FG07826.1 [*Gibberella zeae* PH-1]	2/3%/35 (>33)	K.LTNARLAAR.K (2+)	gi|46126897	25607/5.18		
23	NADP-dependent glutamate dehydrogenase [*Trichophyton rubrum*]	2/5%/49 (>42)	R.EIGYIFGAYR.K (2+) K.LIELGATVVSLSDSK.G (2+)	gi|84993814	49088/5.74	69231/6.46	3.8 ± 0.8
24	NADP-specific glutamate dehydrogenase (NADP-GDH) (NADP-dependent glutamate dehydrogenase)	8/1%/51 (>34)	K.FLGFEQIFK.N (2+)	gi|231989	50141/6.13	68269/6.51	3.9 ± 1.3
	NADP linked glutamate dehydrogenase [*Emericella nidulans*]	8/1%/51 (>34)	K.FLGFEQIFK.N (2+)	gi|2348	49819/6.24		

25	Glyceraldehyde-3-phosphate dehydrogenase [*Pleospora tarda*]	12/9%/51 (>34)	K.VDGNNLTVNGK.T (2+)	gi|32264090	12829/5.81	12759/6.83	136.0 ± 21.4
26	Glyceraldehyde 3-phosphate dehydrogenase [*Stemphylium vesicarium*]	2/10%/54 (>33)	K.VGINGFGR.I (2+) K.VDGNNLTVNGK.T (2+)	gi|64330180	19335/6.64	17069/6.65	3.7 ± 0.7
27	Hypothetical protein BC1G_05706 [*Botryotinia fuckeliana* B05.10]	7/8%/53 (>34)	K.LFIGGLAWHTDENALR.E (2+) K.LFIGGLAWHTDENALR.E (3+)	gi|154313713	18988/5.47	11897/6.83	Unique to Alg3-24
28	Hypothetical protein SNOG_07588 [*Phaeosphaeria nodorum* SN15]	3/6%/52 (>35)	K.GDEVLFLSDTK.T (2+)	gi|111063934	18265/5.46	12586/6.94	16.9 ± 9.6
29	Hypothetical protein FG07949.1 [*Gibberella zeae* PH-1]	5/9%/65 (>36)	K.TLAFLDIGPLSK.G (2+)	gi|46127143	14301/6.06	25453/6.75	25.4 ± 5.8
30	HS30_EMENI 30 KD HEAT SHOCK PROTEIN [*Aspergillus nidulans* FGSC A4]	9/7%/65 (>34)	K.ASLKDGILSLVVPK.A (2+) K.ASLKDGILSLVVPK.A (3+) K.DGILSLVVPK.A (2+)	gi|67524145	20272/6.10	27769/6.79	4.2 ± 0.9
	GTP-binding nuclear protein GSP1/Ran [*Aspergillus terreus* NIH2624]	2/5%/49 (>34)	K.SNYNFEKPFLWLAR.K (2+) K.SNYNFEKPFLWLAR.K (3+)	gi|115390076	26397/7.68		
31	Proteasome component Prs2, putative [*Aspergillus fumigatus* Af293]	4/9%//141 (>35)	K.AITAANITSIGVR.G (2+) K.LIDPSSVSHIFR.L (2+)	gi|70999540	28544/6.84	31923/6.65	4.7 ± 0.5
	Hypothetical protein SNOG_05667 [*Phaeosphaeria nodorum* SN15]	5/9%/136 (>35)	K.AITAANITSLGVR.G (2+) R.IANISQVYTQR.A (2+)	gi|160706740	28215/6.36		
32	Hypothetical protein SNOG_06484 [*Phaeosphaeria nodorum* SN15]	12/29%/302 (>34)	R.DKTLIIWNLTR.D (2+) K.TLIIWNLTR.D (2+) R.LWELSTGVTTR.R (2+) R.RFVGHTNDVLSVSFSADNR.Q (3+) R.FSPNPQNPVIVSAGWDK.L (2+) K.VWELASCR.I (2+)	gi|160707097	24580/8.30	28541/6.82	-8.4 ± 4.3
	G-protein complex beta subunit CpcB [*Aspergillus clavatus* NRRL 1]	12/15%/260 (>34)	R.DKTLIIWNLTR.D (2+) K.TLIIWNLTR.D (2+) R.FSPNPQNPVIVSAGWDK.L (2+) K.VWELASCR.L (2+) K.DGTTMLWDLNESK.H (2+)	gi|121708550	35410/6.20		

33	Hypothetical protein SNOG_09416 [*Phaeosphaeria nodorum* SN15]	3/5%/76 (>35)	K.TFPALFGLHR.N (2+) R.GPAVLDDFTSSYGYK.F (2+)	gi|111062488	56983/6.56	21923/7.29	3.4 ± 0.4
34	Glyceraldehyde-3-phosphate dehydrogenase [*Alternaria japonica*]	14/9%/51 (>35)	K.VDGNNLTVNGK.T (2+)	gi|38604344	12362/5.91	12328/7.21	177.9 ± 36.5
35	Hypothetical protein SNOG_11193 [*Phaeosphaeria nodorum* SN15]	2/11%/52 (>33)	R.SILGATNPLASAPGTIR.G (2+)	gi|111060572	16988/6.84	26585/7.06	15.7 ± 6.7
36	NADP-dependent mannitol dehydrogenase [*Alternaria alternata*]	7/15%/123 (>34)	K.VVIVTGASGPTGIGTEAAR.G (2+) K.LVQDVIKDFGK.V (2+) K.VDVFIANAGK.T (2+)	gi|37780013	29129/5.86	33308/6.96	10.4 ± 3.0
37	Hypothetical protein SNOG_14959 [*Phaeosphaeria nodorum* SN15]	3/10%/63 (>35)	K.GIHATAYSCLGSTDSPLYK.N (3+) K.SVQQVLLMWGLQR.G (2+)	gi|111056382	33651/5.95	33308/7.00	14.5 ± 4.8
38	Hypothetical protein CHGG_00413 [*Chaetomium globosum* CBS 148.51]	5/3%/126 (>34)	R.LQILSPFSAWDGK.D (2+) K.LSHTFNEGQLEWFK.N (2+)	gi|116179570	85505/6.34	29154/7.13	4.5 ± 1.3
39	Hypothetical protein SNOG_05288 [*Phaeosphaeria nodorum* SN15]	9/11%/191 (>34)	K.KLSEFVNVVPVIAK.S (3+) K.KLSEFVNVVPVIAK.S (2+) K.LSEFVNVVPVIAK.S (2+) R.IKEEFQFHNLR.M (2+) R.ELLPFAVVGSER.T (2+)	gi|111066559	38308/7.18	41643/6.96	7.8 ± 3.3
40	Hypothetical protein SNOG_12477 [*Phaeosphaeria nodorum* SN15]	34/11%/217 (>35)	K.IVEFAHSQGQK.I (2+) K.IGIQLGHAGR.K (2+) R.KASTVAPWLSK.G (2+) K.ASTVAPWLSK.G (2+) K.QHIHGGPGYQEPFAK.A (3+) K.QHIHGGPGYQEPFAK.A (2+)	gi|111059170	46457/6.19	44944/7.08	3.3 ± 0.2
	Hypothetical protein An02g01330 [*Aspergillus niger*]	13/5%/116 (>35)	R.VIEFAHSQGQK.I (2+) R.TDEYGGSFENR.M (2+)	gi|145231593	45786/6.69		
	Phosphoglycerate kinase	3/6%/106 (>35)	R.IVGAIPTIK.H (2+) R.AHSSMVGVDLPQK.A (2+) K.ELDYFAK.A (2+)	gi|129928	44720/6.16		

41	Hypothetical protein SNOG_07136 [*Phaeosphaeria nodorum* SN15]	16/18%/308 (>34)	K.LSIADVDLK.G (2+) R.IVGALPTIK.Y (2+) R.AHSSMVGVDLPQK.A (2+) K.IVIIGGGDTATVAAK.Y (2+) K.LSHVSTGGGASLELLEGK.D (3+) K.DLPGVSALSSK.- (2+)	gi|111064667	44853/6.30	43500/7.24	4.7 ± 0.8
	Phosphoglycerate kinase [*Botryotinia fuckeliana* B05.10]	12/16%/271 (>34)	R.VDFNVPLDSDK.N (2+) R.AHSSMVGVDLPQK.A (2+) K.IVIIGGGDTATVAAK.Y (2+) K.LSHVSTGGGASLELLEGK.D (3+) K.DLPGVSALSSK.- (2+)	gi|154295774	44732/6.30		
42	Hypothetical protein SNOG_12004 [*Phaeosphaeria nodorum* SN15]	13/16%/297 (>34)	R.SGCTVIIPFR.E (2+) R.SGCTVIIPFREEMAK.R (3+) R.SGCTVIIPFREEMAK.R (2+) R.HSDIVYNLIGR.D (2+) R.SIYPETTIVRPAPMFGFEDR.L (3+) K.DLDIEPVELK.S (2+) K.KYLHVLDDQ.- (2+) K.YLHVLDDQ.- (2+)	gi|111059296	43951/6.85	42571/7.25	5.9 ± 1.1
	Glutathione-dependent formaldehyde dehydrogenase [*Coccidioides immitis* RS]	3/8%/175 (>34)	R.IQIYYTGVCHTDAYTLSGK.D (3+) R.KFGATDFVNPTK.L (2+) K.FGATDFVNPTK.L (2+) R.VVFMEYDLR.N (2+)	gi|119191642	41185/6.71		
	NADH-ubiquinone oxidoreductase 40 kDa subunit, mitochondrial precursor [*Coccidioides immitis* RS]	5/7%/172 (>34)	R.NTQSIEESVR.H (2+) K.KYLHVLDDQ.- (2+) K.YLHVLDDQ.- (2+)	gi|119194685	44401/6.85		

43	Hypothetical protein SNOG_09845 [*Phaeosphaeria nodorum* SN15]	26/22%/ 465 (>32)	R.DGMSFLDLSVR.Q (2+) K.KYEGHNIDILTFNQSR.Y (3+) K.KYEGHNIDILTFNQSR.Y (2+) K.AEYIMELTDK.T (2+) K.GGTIIDYEGSVR.L (2+) R.LLEIAQVPK.E (2+) K.RVVEANELAMEIIPNGK.S (3+) R.VVEANELAMEIIPNGK.S (2+) K.KGEADISVLQLETAVGAAIK.H (3+) K.SDLYTLQHGQLVIDPNR.F (3+) K.SDLYTLQHGQLVIDPNR.F (2+) K.IVELDHLTITGPVNLGR.G (3+) K.IVELDHLTITGPVNLGR.G (2+)	gi|160702723	62177/7.01	56731/7.17	6.2 ± 1.0
	Hypothetical protein BC1G_10557 [*Botryotinia fuckeliana* B05.10]	10/11%/233 (>32)	R.DGMSFLDLSVR.Q (2+) K.KYEGHNIDILTFNQSR.Y (3+) K.SEYIMELTDK.T (2+) K.GGTIIDYEGSVR.L (2+) R.LLEIAQVPK.E (2+)	gi|154301169	58687/6.55		
44	Hypothetical protein SNOG_09845 [*Phaeosphaeria nodorum* SN15]	57/22%/511 (>33)	R.DGMSFLDLSVR.Q (2+) K.KYEGHNIDILTFNQSR.Y (3+) K.KYEGHNIDILTFNQSR.Y (2+) K.YEGHNIDILTFNQSR.Y (2+) K.AEYIMELTDK.T (2+) K.GGTIIDYEGSVR.L (2+) R.LLEIAQVPK.E (2+) R.VVEANELAMEIIPNGK.S (2+) K.KGEADISVLQLETAVGAAIK.H (3+) K.SDLYTLQHGQLVIDPNR.F (3+) K.IVELDHLTITGPVNLGR.G (3+)	gi|160702723	62177/7.01	54808/7.52	4.5 ± 1.1
	Hypothetical protein BC1G_10557 [*Botryotinia fuckeliana* B05.10]	32/11%/234 (>33)	R.DGMSFLDLSVR.Q (2+) K.KYEGHNIDILTFNQSR.Y (3+) K.KYEGHNIDILTFNQSR.Y (2+) K.YEGHNIDILTFNQSR.Y (2+) K.SEYIMELTDK.T (2+) K.GGTIIDYEGSVR.L (2+) R.LLEIAQVPK.E (2+)	gi|154301169	58687/6.55		

45	Hypothetical protein SNOG_04712 [*Phaeosphaeria nodorum* SN15]	20/14%/341 (>34)	K.FPGMAITYALMQR.F (2+) K.VQVHPTGLVDPK.D (2+) R.GEGGILLNNK.G (2+) R.LGGSSLLGCVVYGR.V (2+) K.KDDLWIAVK.G (2+) K.YAPEIVIGR.V (2+) R.VKGQEVTLEY.- (2+)	gi|111067352	69354/6.30	70192/7.05	3.5 ± 0.6
	Hypothetical protein SS1G_06222 [*Sclerotinia sclerotiorum* 1980]	21/10%/226 (>34)	K.FPGMAITYALMQR.L (2+) K.VQVHPTGLVDPK.D (2+) K.ASNVLDFHTR.H (2+) R.LGGSSLLGCVVYGR.V (2+) R.LGQISLHIDPSQPGK.I (2+)	gi|156054748	68242/6.36		
46	Unnamed protein product [*Aspergillus oryzae*]	6/5%/173 (>34)	K.FIEKEIEDVVK.I (2+) R.DDVHQSVQAQQLALALR.D (3+) R.DDVHQSVQAQQLALALR.D (2+) R.HIGPGVYDIHSPR.V (2+) R.HIGPGVYDIHSPR.V (3+)	gi|83769201	87338/6.48	90000/7.15	21.1 ± 6.6
	Cobalamin-independent methionine synthase [*Epichloe festucae*]	23/5%/165 (>34)	K.LLPVYEQLLVK.L (2+) R.DDVHQSVQAQQLALALR.D (3+) R.DDVHQSVQAQQLALALR.D (2+) R.HIGPGVYDIHSPR.V (2+) R.HIGPGVYDIHSPR.V (3+)	gi|34500101	77555/6.31		
47	Unamed protein product [*Aspergillus oryzae*]	7/6%/240 (>34)	K.FIEKEIEDVVK.I (2+) K.GMLTGPITCLR.W (2+) R.DDVHQSVQAQQLALALR.D (3+) R.DDVHQSVQAQQLALALR.D (2+) R.HIGPGVYDIHSPR.V (2+) R.HIGPGVYDIHSPR.V (3+)	gi|83769201	87338/6.48	87500/6.93	-8.0 ± 1.3
	Cobalamin-independent methionine synthase [*Epichloe festucae*]	19/5%/173 (>34)	K.LLPVYEQLLVK.L (2+) R.DDVHQSVQAQQLALALR.D (3+) R.DDVHQSVQAQQLALALR.D (2+) R.HIGPGVYDIHSPR.V (2+) R.HIGPGVYDIHSPR.V (3+)	gi|34500101	77555/6.31		
48	Conserved hypothetical protein [*Coprinopsis cinerea* okayama7#130]	34/14%/112 (>36)	R.IIPNFMLQGGDFTR.G (2+) K.FADENFQLK.H (2+)	gi|116499218	17509/9.19	12500/7.92	776.2 ± 446.8

49	Hypothetical protein SNOG_00050 [*Phaeosphaeria nodorum* SN15]	46/6%/69 (>36)	R.VIPDFMLQGGDFTR.G (2+)	gi|111070425	24452/9.25	17778/7.29	-3.0 ± 0.8
50	Hypothetical protein SNOG_00050 [*Phaeosphaeria nodorum* SN15]	46/6%/69 (>36)	R.VIPDFMLQGGDFTR.G (2+)	gi|111070425	24452/9.25	24038/8.65	5.6 ± 1.6
	Peptidyl-prolyl cis-trans isomerase [*Ajellomyces capsulatus* NAm1]	46/5%/69 (>36)	R.VIPDFMLQGGDFTR.G (2+)	gi|154279664	26409/9.34		
51	Hypothetical protein SNOG_05974 [*Phaeosphaeria nodorum* SN15]	43/22%/367 (>34)	K.LSLYDIR.L (2+) R.LAPGVAADIGHINTK.S (2+) K.GAEIVVIPAGVPR.K (2+) R.DDLFNTNASIVR.D (2+) R.VQFGGDEVVKAK.D (2+) K.DGAGSATLSMAMAGAR.F (2+)	gi|160706863	35963/9.03	33656/9.06	-22.9 ± 15.2
	Conserved hypothetical protein [*Botryotinia fuckeliana* B05.10]	26/11%/215 (>34)	R.DDLFNTNASIVR.D (2+) R.VQFGGDEVVQAK.D (2+) K.DGAGSATLSMAMAGAR.M (2+)	gi|154309029	35646/7.75		
52	Hypothetical protein SNOG_05974 [*Phaeosphaeria nodorum* SN15]	43/22%/367 (>34)	K.LSLYDIR.L (2+) R.LAPGVAADIGHINTK.S (2+) K.GAEIVVIPAGVPR.K (2+) R.DDLFNTNASIVR.D (2+) R.VQFGGDEVVKAK.D (2+) K.DGAGSATLSMAMAGAR.F (2+)	gi|160706863	35963/9.03	35033/9.06	-16.0 ± 9.2
	Conserved hypothetical protein [*Sclerotinia sclerotiorum* 1980]	26/11%/215 (>34)	R.DDLFNTNASIVR.D (2+) R.VQFGGDEVVQAK.D (2+) K.DGAGSATLSMAMAGAR.M (2+)	gi|156032838	35708/8.48		

53	Hypothetical protein SNOG_12110 [*Phaeosphaeria nodorum* SN15]	43/30%/524 (>35)	K.SLVDTDNEVAQIMEK.E (2+) R.ESILLIASENVTSR.A (2+) R.AVFDALGSPMSNK.Y (2+) R.LMGLDLPHGGHLSHGYQTPQR.K (3+) R.KISAVSTYFETFPYR.V (3+) R.KISAVSTYFETFPYR.V (2+) K.VLVAGTSAYCR.E (2+) K.SPFPYCDIVTTTTHK.S (3+) K.SPFPYCDIVTTTTHK.S (2+) K.LVTDGTDNHMVLIDLKPFALDGAR.V (3+) R.VEAVLEQVNIACNK.N (2+)	gi|111059402	52643/7.17	49337/7.62	-3.1 ± 0.6
	Serine hydroxymethyltransferase [*Sclerotinia sclerotiorum* 1980]	37/15%/283 (>35)	R.ESIILIASENVTSR.A (2+) R.AVFDALGSPMSNK.Y (2+) R.LMGLDLPHGGHLSHGYQTPQR.K (3+) R.KISAVSTYFETFPYR.V (3+) R.KISAVSTYFETFPYR.V (2+) K.VLVAGTSAYCR.L (2+)	gi|156039359	52966/7.21		
54	Enolase (2-phosphoglycerate dehydratase) (2-phospho-D-glycerate hydro-lyase)	4/13%/228 (>32)	R.GNPTVEVDIVTETGLHR.A (2+) K.AVANVNDTIAPALIK.E (2+) K.DQSAVDAFLNK.L (2+) K.TNLGANAILGVSMAIAK.A (2+)	gi|14423684	47347/5.19	13276/8.42	73.8 ± 37.7

a) Proteins were identified using ESI-quadrupole-TOF MS/MS data.

b) Access ID is the access identification of the protein from the non-redundant database search.

c) Fold-change refers to the mean difference in intensity of the matched protein spots in Alg3-24 relative to 90-2.

d) Number of peptides matched.

e) Ion score is −10 log (P), where P is probability that the observed match is a random event; individual ion scores larger than the threshold values in parentheses indicates identity or extensive homology ($p < 0.05$).

abundant in 90-2. Two proteins (spots 1 and 27) were found to be unique in Alg3-24. Among the spots identified by MS/MS, 24 matched proteins with known biological functions, including proteins involved in energy metabolism (8 spots), cell rescue, defense and virulence (7 spots), general metabolism (6 spots), protein synthesis (1 spot), protein fate (1 spot), cellular communication (1 spot), and 30 unclassified proteins.

A.3.4.2. Energy metabolism

Three spots corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spots 25, 26, 34), an enzyme catalyzing the NAD-dependent conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate in the glycolytic pathway, were found to be significantly more abundant in the mycelium of Alg3-24 compared to that of 90-2 (Fig. A-4; Table A-2). GAPDH plays an important role in cellular energy production and is also involved in suppression of reactive oxygen species induced by heat shock (Baek *et al.*, 2008). The enzyme is considered a virulence factor in *E. coli*, since extracellular GAPDH was found only on the surface of pathogenic *E. coli* cells but not on nonpathogenic cells (Egea *et al.*, 2007). Similarly, a proteomic comparison of strains of the phytopathogenic fungus *Botrytis cinerea* indicated that GAPDH was present only in the more virulent strain of the fungus (Fernández-Acero *et al.*, 2007). Pancholi and Chhatwal (2003) noted that GAPDH is one of a number of housekeeping enzymes that can act as virulence factors in fungal and bacterial species. The identification of greater quantities of this enzyme in Alg3-24 relative to 90-2 could indicate that GAPDH also has a role in the pathogenicity of *P.*

tritici-repentis, although further research is required to test this hypothesis. A more active oxidative metabolism may also contribute to increased virulence in Alg3-24.

Spots matching two other glycolytic enzymes, phosphoglycerate kinase (PGK, spots 40, 41) and enolase (spot 54), were also found to be significantly more abundant in Alg3-24 than in 90-2 (Fig. A-4; Table A-2). Accumulation of PGK was greater in the symbiotic versus asymbiotic stage of the mycorrhizal fungus *Glomus* (Harrier and Sawczak, 2003), and disruption of the *pgk* gene encoding PGK resulted in poor growth and reduced formation of conidia in *Aspergillus nidulans* (Streatfield and Roberts, 1993). Thus, increased levels of this enzyme in Alg3-24 may contribute to its enhanced pathogenic ability. Moreover, Pancholi and Chhatwal (2003) hypothesized that PGK may serve as a virulence factor in microorganisms causing human diseases, especially given its presence on the tegument of certain protozoa and other parasites. The intensity of the spot corresponding to enolase was 73.8-fold higher in the mycelium of Alg3-24 than in 90-2 (Fig. A-4; Table A-2). In addition to its glycolytic role, enolase participates in a variety of other cellular activities, acting as a HSP, binding cytoskeletal and chromatin structures to modulate transcription, and playing a crucial role in pathophysiological processes (Pancholi, 2001). An *in vivo* increase in the expression of the enolase gene was shown to contribute to the pathogenesis of *Aeromonas hydrophila,* which causes gastroenteritis in humans (Sha *et al.*, 2003). However, to our knowledge, the role of enolase in fungal

phytopathogenicity has not been investigated, despite its contribution to microbial virulence reported in the medical literature.

Transaldolase (spot 18), an enzyme of the pentose phosphate pathway, was upregulated in the mycelium of 90-2 relative to Alg3-24 (Fig. A-4; Table A-2). However, a study with a transaldolase deletion mutant of yeast revealed that glucose could still be metabolized through this pathway without affecting the yeast phenotype (Schaaff-Gerstenschlager and Zimmerman, 1993), suggesting that a lower abundance of transaldolase in Alg3-24 may not have a significant effect on its physiology. NADP-dependent mannitol dehydrogenase (spot 36), an important *A. alternata* allergen (Schneider *et al.*, 2006), was more abundant in Alg3-24 than in 90-2 (Fig. A-4; Table A-2).

A.3.4.3. Cell rescue, defense and virulence

Three HSPs (spots 2, 19, 30) were found to be significantly more abundant in the mycelium of Alg3-24 relative to 90-2 (Fig. A-4; Table A-2). The level of a protein matching an HSP70 from *Magnaporthea grisea* (spot 2) was 8.7-fold higher in Alg3-24 than in 90-2 (Fig. A-4; Table A-2). HSP70 molecular chaperones are present in all cells and are essential for tolerance to protein denaturing stresses as well as for protein biogenesis and regulation under normal growth conditions (Shaner *et al.*, 2005). It was found that lower levels of transcription of the *hsp70* gene at 37^{o}C were correlated with greater temperature sensitivity and reduced virulence in the fungus *Histoplasma capsulatum* (Caruso *et al.*, 1987). Heat shock and up-regulation of a plant HSP70 also facilitated infection of coffee leaves by *Colletotrichum gloeosporioides* (Chen *et al.*, 2003),

and heat also induced susceptibility of soybean to fungal infection. While these results do not necessarily indicate an increase in HSPs in the infecting pathogens, fungal virulence appears to be increased under heat-shock conditions. Therefore the greater abundance of HSP70 in the mycelium of Alg3-24 may reflect a greater thermotolerance in this isolate, perhaps contributing to superior pathogenic fitness relative to 90-2, especially under fluctuating conditions in the field. Two proteins matching HSP30 (spots 19, 30) were also found to be significantly altered in abundance between Alg3-24 and 90-2 (Fig. A-4; Table A-2). HSP30 has been found to be a membrane protein in *S. cerevisiae*, and is induced by heat shock, ethanol, and exposure to weak organic acid (Seymour and Piper, 1999).

Protein spots matching the ER chaperone BiP (spots 4, 5) were also significantly more intense in the mycelium of Alg3-24 compared to that of 90-2 (Fig. A-4; Table A-2). BiP proteins are molecular chaperones that are present in the endoplasmic reticulum (ER) and belong to the HSP70 family. Although BiP proteins are constitutively synthesized, their expression is also induced by various stresses that cause the accumulation of unfolded proteins in the ER lumen (Kozutsumi *et al.*, 1988; Van Gemeren *et al.*, 1997). A deduced amino acid sequence of the BiP protein encoded by the *bipA* gene from *A. oryzae* contained three HSP70 conserved sequence motifs (Kasuya *et al.*, 1999), and its expression complemented a temperature-sensitive, slow growth mutant that had defects in karyogamy and protein translocation into the ER lumen (Kasuya *et al.*, 1999). The expression of the *Aspergillus niger bipA* gene was also increased by heat shock (Kozutsumi *et al.*, 1988). Thus, the greater abundance of ER chaperone BiP found

in Alg3-24, combined with the enhanced levels of the related HSP70, may bestow this isolate with a greater tolerance for temperature fluctuations or other stresses, aiding in persistence in the host.

In addition to HSPs and related proteins, two other spots matching stress-related proteins were found to be significantly more abundant in Alg3-24 versus 90-2 (Fig. A-4; Table A-2). One matched glutathione-dependent formaldehyde dehydrogenase (spot 42), an enzyme involved in the detoxification of formaldehyde (Nakagawa *et al.*, 2004), and the other matched a peptidyl-prolyl cis/trans isomerase (spot 50). Peptidyl-prolyl cis/trans isomerases are transcriptionally induced in response to unfolded proteins in the ER, catalyzing the cis/trans isomerisation of a prolyl bond (Pemberton, 2006). The contribution of these proteins to pathogenic ability in Alg3-24 is not clear, although they likely also reflect enhanced stress tolerance in this isolate.

A.3.4.4. Metabolism

Six spots matching metabolism-related proteins were found to be differentially abundant in the mycelia of Alg3-24 and 90-2, including a putative amidase family protein (spot 22) and five proteins with roles in amino acid metabolism (Fig. A-4; Table A-2). The latter included two spots matching NADP-dependent glutamate dehydrogenase (spots 23, 24), an enzyme that catalyzes the reversible amination of 2-oxoglutarate to form glutamate (Botton and Dell, 1994) and which is believed to be dispensable in ectomycorrhizal fungi (Morel *et al.*, 2006). Two spots matched cobalamin-independent methionine synthase (spots 46, 47), which catalyzes the transfer of a methyl group from tetrahydrofolate to

homocysteine to produce methionine (Suliman et al, 2007), and one spot matched serine hydroxymethyltransferase (spot 53), which is involved in serine metabolism (Schlüepen *et al.*, 2003).

A.3.4.5. Other mycelial proteins

The abundance of translation elongation factor (spot 7) was 5.2-fold greater in the mycelium of Alg3-24 than in 90-2 (Fig. A-4; Table A-2). This protein is involved in several biological processes, including protein synthesis, cell proliferation, apoptosis and tumorigenesis (Yang *et al.*, 2008). A spot corresponding to putative proteasome component Prs2 (spot 31), which has a role in protein processing, was also more abundant in Alg3-24 relative to 90-2. In contrast, the G-protein complex beta subunit CpcB (spot 32), which is involved in cellular communication, was down-regulated in Alg3-24. The remaining 30 spots identified as being differentially abundant in the mycelia of Alg3-24 and 90-2 were mostly hypothetical or unclassified proteins, two of which were unique to Alg3-24.

A.4. Concluding remarks

Despite the observation that the form of *ToxB* found in race 4 isolate 90-2 of *P. tritici-repentis* is an expressed gene (Amaike *et al.*, 2008), the encoded protein is not produced in detectable amounts (Fig. A-2). This appears to support the suggestion by Andrie *et al.* (2008) that the homolog of *ToxB* in race 4 may be evolving towards a fate as a pseudogene. However, it is not clear why, if this is the case, avirulent isolates from geographically distant locations would maintain identical forms of the gene; *ToxB* from isolate 90-2 (collected in Manitoba) is

only 86% homologous to wild-type *ToxB* from virulent isolates, but it is identical to *toxb* reported for another avirulent isolate, SD20, collected in South Dakota (Martinez *et al.*, 2004). This suggests that the *ToxB* homologs of race 4 are under similar evolutionary pressures (Strelkov *et al.*, 2006). However, the role of these homologs, and those recently reported in other ascomycetes (Andrie *et al.*, 2008), remains undefined.

Beyond the differences observed with respect to Ptr ToxB (Fig. A-2), analysis of the full mycelial and secreted proteomes of Alg3-24 and 90-2 revealed major differences between virulent and avirulent isolates. Fungal pathogenicity requires the coordinated regulation of multiple genes (and their protein products) involved in host recognition, spore germination, hyphal penetration, appressorium formation, toxin production and secretion. We identified a number of proteins that were more abundant in Alg3-24 versus 90-2 and which could contribute to an enhanced basic pathogenic ability in the former. These included the secreted enzymes α-mannosidase and exo-β-1,3-glucanase (Table A-1; Fig. A-3), which may help degrade host cell walls, facilitating hyphal penetration and maceration of leaf tissue. Similarly, the greater abundance of HSP70 and the related BiP proteins in the virulent isolate (Table A-2; Fig. A-4) may contribute to enhanced stress tolerance during the infection process and within the host, especially given that these proteins have been implicated in microbial virulence in other pathosystems. A number of metabolic enzymes, including GAPDH, PGK and enolase, have also been reported to have roles as pathogenicity and/or virulence factors in other fungal and bacterial species, and were found to be more abundant

in the virulent race 5 isolate of *P. tritici-repentis* (Fig. A-4; Table A-2). The proteome-level differences, combined with the previous observation that significantly lower numbers of appressoria were formed by an avirulent isolate of the fungus (Amaike *et al.*, 2008), suggest a reduced general pathogenic ability in some isolates of *P. tritici-repentis*, irrespective of toxin production. Such differences could reflect an adaptation to a saprophytic habit in race 4 isolates of the fungus.

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