AN ABSTRACT OF THE DISSERTATION OF

Rachael M. Andrie for the degree of Doctor of Philosophy in Botany and Plant Pathology, presented on April 21, 2006.

Title: Examination of the Role Host-Selective Toxins Play in Fungal-Plant Interactions.

Abstract approved:

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Lynda M. Ciuffetti

Pyrenophora tritici-repentis, causal agent of tan spot of wheat, produces multiple host-selective toxins (HSTs), including Ptr ToxA, encoded for by ToxA, Ptr ToxB, encoded for by ToxB, and Ptr ToxC. Variable distribution of these three HSTs among different isolates of P. tritici-repentis, both singularly and in all possible combinations, defines a complex race structure for this fungus. Eight races have been formally published. P. tritici-repentis race identification is typically based on inoculation of a standard set of host differentials. However, we found that a combination of phenotypic and genotypic characterization can be used to designate new races of P. tritici-repentis, and in some cases, is necessary to reveal variability not apparent by phenotype alone. In fact, genotypic and phenotypic characterization revealed two new races, which we designated race 9 and race 10. To aid in race classification, we utilized the association between HST production and race identity to develop a multiplex PCR assay based on HST gene-specific primers. Application of our multiplex PCR assay revealed that ToxB homologs, designated PbToxB, are present in P. bromi, causal agent of brown spot of bromegrass. Southern analysis of P. tritici-repentis and its Pleosporalean relatives anchored in a phylogram suggested that the distribution of ToxB extends to other plant pathogens in the Pyrenophora and Pleosporaceae. A search of available fungal genomes identified a distant homolog in Magnaporthe grisea, causal agent of rice blast. Due to
the relatedness of *P. tritici-repentis* and *P. bromi*, and that of their grass hosts, we sought
to determine if the *ToxB* homologs in *P. bromi* act as HSTs in brownspot of brome grass.
Though Pb *ToxB* does not act as an HST for *P. bromi* on tested brome grass, evidence
suggests *PbToxB* may function for *P. bromi* on tested wheat differentials. Alternatively,
variation in functional homologs of *ToxB* may represent evolutionary remnants of an
arms race between ascomycetes and their grass hosts. Several valuable tools were
developed as a result of this work. In addition to multiplex PCR, we constructed a set of
fluorescent protein expression vectors that we anticipate will be useful for a variety of
applications in fungal-related research areas.
Examination of the Role Host-Selective Toxins Play in Fungal-Plant Interactions

by

Rachael M. Andrie

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Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Chair of the Department of Botany and Plant Pathology

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Rachael M. Andrie, Author
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CONTRIBUTION OF AUTHORS

Dr. Lynda Ciuffetti was involved in the design and editing of all chapters. Preliminary work by Dr. Iovanna Pandelova established the foundation for Chapter 2. Chapter 3 was made possible by the expertise of Dr. Joey Spatafora and the members of his laboratory. Finally, Rebecca Tippner-Hedges assisted with data collection for Chapter 3 and Dr. J. Patrick Martinez constructed the ToxB promoter-driven GFP vector used in Chapter 5.
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This work was completed in loving memory of

Dr. Marwood Emery Wegner,
who would be more proud of me than I can imagine,
and
Doris Ann Wegner,
whom I was never fortunate enough to meet.
Examination of the Role Host-Selective Toxins Play in Fungal-Plant Interactions

Chapter 1

Introduction

Tan spot of wheat

Tan spot of wheat is caused by the fungal ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* [Died.] Shoem.) (Hosford 1971). Prior to the establishment of wheat as its primary host, grasses other than cereals were considered the main hosts of this fungus (De Wolf et al. 1998). *P. tritici-repentis* was first identified on grass hosts, particularly the grass *Agropyrens repens* (Diedicke 1902), and has subsequently been identified on over 25 grass species, with reactions ranging from susceptible to resistant (Ali and Francl 2003a, De Wolf et al. 1998, Krupinsky 1982, 1992). Although *P. tritici-repentis* had also been identified on wheat as early as the late 1920s (Nisikado 1928) to early 1930s (Conners 1937, Mitra 1934), it was not until the late 1960s (Hosford 1971) to early 1970s (Hosford 1982) that major tan spot epidemics emerged. Since that time, *P. tritici-repentis* has been identified in all major wheat growing areas of the world (Strelkov and Lamari 2003) and tan spot of wheat is credited for major yield losses (Ciuffetti and Tuori 1999, Hosford 1982).

The emergence of tan spot as a major wheat disease in the 1970s has been attributed to changes in cultural practices from stubble burning and conventional tillage to conservation- and zero-tillage, shorter crop rotations and continuous wheat cultivation, and the culture of highly susceptible cultivars (Ciuffetti and Tuori 1999, De Wolf et al. 1998, Strelkov and Lamari 2003). *P. tritici-repentis* reproduces both sexually as ascospores, which overwinter in wheat stubble and are generally thought to serve as primary inoculum to start the disease cycle in spring (Hosford 1971), and asexually as conidia, which have also been credited as primary inoculum (Krupinsky 1992), as well as provide secondary inoculum to perpetuate the disease cycle throughout the growing

*P. tritici-repentis* produces two distinct symptoms on susceptible wheat, necrosis and chlorosis. Depending upon the host genotype and isolate of *P. tritici-repentis* tested, these symptoms are observed as well-defined tan necrotic lesions, spreading chlorosis, or a combination of both symptoms on one leaf (Lamari and Bernier 1989a). Resistant reactions to *P. tritici-repentis* are characterized by small, brown necrotic flecks (Lamari and Bernier 1989a). Based on these observations, Lamari and Bernier (1989a) categorized isolates of *P. tritici-repentis* into four pathotypes based on the ability to produce necrosis and/or chlorosis on wheat. Pathotype 1 isolates produce both necrosis and chlorosis; isolates of pathotype 2 and 3 produce only one symptom each, necrosis or chlorosis, respectively; and, nonpathogenic pathotype 4 isolates lack either symptom. Due to the emergence of additional variation in *P. tritici-repentis*, Lamari and colleagues (1995) proposed a race designation system modeled after that of cereal rusts to describe the isolates of *P. tritici-repentis*. The four pathotypes previously described became races 1 through 4, and a new isolate was designated as race 5. The new race-based categorization system is limited only by the size and effectiveness of the differential set, in contrast to the original pathotype classification system that allowed the description of only four pathotypes. The virulence patterns of the various races of *P. tritici-repentis* have since been discovered to be toxin-based in that each compatible interaction between
an isolate and its corresponding susceptible host differential is mediated by a host-
selective toxin (Strelkov and Lamari 2003).

Pyrenophora tritici-repentis produces multiple host-selective toxins (HSTs)

Host-selective toxins (HSTs), a diverse set of molecules known only to be
produced by plant pathogenic fungi, have been shown to function as essential disease
determinants in a number of plant host-pathogen interactions (reviewed in Markham and
Hille 2001, Walton 1996, Wolpert et al. 2002). They are implicated in
pathogenesis/virulence because they are toxic only to hosts susceptible to the fungus, and
in turn, toxin production by the pathogen is strictly correlated with disease elicitation on a
susceptible host. The availability of molecular approaches for use in fungi allowed for
the definitive confirmation of HST causality in disease for a number of pathosystems
Yoder et al. 1997). Species or pathotypes in just two genera, Cochliobolus and
Alternaria, account for the majority of HSTs produced, most of which are low-molecular
weight products of multifunctional enzymes or complex enzymatic pathways encoded by
complex gene clusters (Walton 1996, Wolpert et al. 2002). However, P. tritici-repentis
produces multiple, distinct HSTs, at least two of which are proteins (Strelkov and Lamari
2003).

Ptr ToxA

Ptr ToxA (Ballance et al. 1989, Tomas et al. 1990, Tuori et al. 1995) (Synonyms:
Ptr necrosis toxin, Ptr toxin and ToxA [Ciuffetti et al. 1998]), the first protein HST
identified, is the best characterized HST produced by P. tritici-repentis. This 13.2 kDa
protein (Tuori et al. 1995) is produced by the most common races of P. tritici-repentis
2003) and is responsible for typical tan spot lesions (Ballance et al. 1989, Tomas et al.
1990, Tuori et al. 1995). Infiltration of Ptr ToxA alone reproduces necrotic symptoms of disease (Ballance et al. 1989, Tomas et al. 1990, Tuori et al. 1995, 2000). Ptr ToxA is encoded for by ToxA, a single copy gene found only in pathogenic isolates that produce the toxin (Ballance et al. 1996, Ciuffetti et al. 1997). The ToxA ORF encodes for a 178 amino acid (aa) residue pre-pro-protein (Ciuffetti et al. 1997). The pre-region is composed of a 23 aa residue secretion signal, consistent with secretion of this HST (Ciuffetti et al. 1997). The 38 aa pro-region (also referred to as the N-domain) consists of a 4.3 kDa anionic domain that is required for proper folding of the mature, secreted 118 aa residue Ptr ToxA protein (also referred to as the C-domain) (Ciuffetti et al. 1997, Tuori et al. 2000). The formation of a disulfide bond was also shown to be required for stability of the active, native form of Ptr ToxA (Tuori et al. 2000). Transformation of a nonpathogenic P. tritici-repentis race 4 isolate with the ToxA gene demonstrated that expression of ToxA is both necessary and sufficient for pathogenesis on tested Ptr ToxA-sensitive wheat cultivars (Ciuffetti et al. 1997). Significant advances into the functional characterization of Ptr ToxA are discussed below.

Ptr ToxB

Ptr ToxB (Synonym: Ptr chlorosis toxin [Ciuffetti et al. 1998]) was the second proteinaceous HST identified. This 6.6 kDa protein HST is responsible for symptoms of spreading chlorosis on sensitive wheat (Orolaza et al. 1995, Strelkov et al. 1999). In contrast to Ptr ToxA, which is encoded for by a single gene, Ptr ToxB is encoded for by the multicopy ToxB gene (Lamari et al. 2003, Martinez et al. 2004, Martinez et al. 2001, Strelkov et al. 2002, Strelkov et al. 2006). The ToxB ORF encodes for an 87 aa residue pre-protein, which is processed to 61 aa at maturity following cleavage of the 23 aa signal sequence upon protein secretion (Martinez et al. 2001). Although it is not yet known if all ToxB loci are actively transcribed, inoculation of nonpathogenic P. tritici-repentis race 4 transformants containing variable numbers of ToxB under the control of the same constitutive promoter revealed a positive correlation between ToxB copy number and chlorotic symptom development (Martinez and Ciuffetti, unpublished data). Thus,
expression of multiple copies of ToxB, or at least high levels of Ptr ToxB production, is both necessary and sufficient for pathogenesis on tested Ptr ToxB-sensitive wheat cultivars. Evolutionary considerations regarding Ptr ToxB are discussed below.

**Ptr ToxC**

Another chlorosis-inducing HST was purified from culture filtrates of isolates of *P. tritici-repentis* that cause chlorosis on a wheat line distinct from that sensitive to Ptr ToxB (Lamari et al. 1995). Unlike Ptr ToxA and Ptr ToxB, it is not proteinaceous in nature. Rather, similar to other known HSTs, Ptr ToxC appears to be a nonionic, polar, low molecular weight molecule (Effertz et al. 2002). Characterization of the gene(s) responsible for its production awaits its purification.

**Other HSTs**

Other toxins have also been suggested to be produced by *P. tritici-repentis*, although these await full characterization (Ali and Franc! 2002, Ciuffetti et al. 2003, Meinhardt et al. 2003, Tuori et al. 1995). For example, characterization of yet another proteinaceous HST, tentatively named Ptr ToxD (Ciuffetti et al. 2003), is currently ongoing in our laboratory. Thus far, Ptr ToxD activity from culture filtrates has been purified to a 10 kDa protein band as determined by SDS-PAGE and confirmed by MALDI mass spectrometry (Pandelova and Ciuffetti 2005). Peptide sequences obtained from a trypsin digest of the 10 kDa protein were separated with a Waters CapLC system and subsequently analyzed by electrospray ionization (ESI) quadruple time-of-flight (Q-TOF) mass spectrometry. Sequences obtained were used to design degenerate primers for use in reverse transcriptase-polymerase chain reaction (RT-PCR). A partial cDNA clone obtained from RT-PCR was used to clone the gene encoding for Ptr ToxD and confirmation that it encodes a HST is in progress. Additionally, in a community effort that is currently in progress in our laboratory in collaboration with the Broad Institute (Galagan et al. 2005), completion of the genomic sequence of a race 1 isolate and the
production of EST libraries from additional races promises to significantly increase the discovery rate of pathogenicity/virulence factors produced by this fungus.

**Complex race structure of *Pyrenophora tritici-repentis***

The specific complement of HSTs produced by a particular isolate of *P. tritici-repentis* determines its host cultivar specificity, and each unique specificity profile defines a unique race. It has been shown that Ptr ToxA causes necrosis on Glenlea and Katepwa (Ballance et al. 1989, Tomas et al. 1990, Tuori et al. 1995); Ptr ToxB causes chlorosis on 6B662 and Katepwa (Orolaza et al. 1995, Strelkov et al. 1999); and Ptr ToxC causes chlorosis on 6B365 (Effertz et al. 2002). The wheat cultivars Salamouni (De Wolf et al. 1998, Strelkov and Lamari 2003) and Auburn (Ciuffetti et al. 1997, Tomas and Bockus 1987, Tomas et al. 1990, Tuori et al. 1995) are resistant to all known races of *P. tritici-repentis* and insensitive to its known toxins. A complex race structure for this fungus is defined by the variable distribution of Ptr ToxA, Ptr ToxB, and Ptr ToxC among different isolates of *P. tritici-repentis*, both singly and in all possible combinations, as visualized by the differential induction of necrosis and/or chlorosis on this standard set of wheat differentials. Eight races, 1-8, each with a unique complement of Ptr ToxA, Ptr ToxB, and Ptr ToxC, and a correspondingly unique host range, have been formally described (Lamari and Bernier 1989a, Lamari et al. 1995, Lamari et al. 2003, Strelkov et al. 2002). Races 1, 2, 7, and 8 produce Ptr ToxA; races 5, 6, 7, and 8 produce Ptr ToxB; and races 1, 3, 6, and 8 produce Ptr ToxC. Nonpathogenic race 4 isolates do not produce any of these three HSTs. As races are currently defined on a limited differential set based on the phenotypic results of inoculation, some *P. tritici-repentis* diversity is likely being overlooked. Indeed, additional races and HSTs are currently under investigation (Ali and Franci 2002, Ali et al. 2002, Ciuffetti et al. 2003, Meinhardt et al. 2003, Chapter 2).
Host reaction to *Pyrenophora tritici-repentis* and its HSTs

Sensitivity of wheat to Ptr ToxA, Ptr ToxB, and Ptr ToxC is dominant to resistance, and is conferred by independent single dominant genes for each toxin (Anderson et al. 1999, Effertz et al. 2002, Faris et al. 1996, Gamba and Lamari 1998, Gamba et al. 1998, Lamari and Bernier 1989c, Orolaza et al. 1995, Stock et al. 1996). Additionally, toxin sensitivity has been shown to cosegregate with host susceptibility to pathogenic races that produce these three HSTs (Gamba and Lamari 1998, Gamba et al. 1998, Lamari and Bernier 1989c, Orolaza et al. 1995, Stock et al. 1996). Therefore sensitivity and susceptibility appear to be controlled by the same loci. *Tsn1* (Anderson et al. 1999, Faris et al. 1996, Stock et al. 1996), *Tsc1* (Effertz et al. 2002), and *Tsc2* (Friesen and Faris 2004), the loci in wheat controlling sensitivity to Ptr ToxA, Ptr ToxC, and Ptr ToxB, respectively, and correspondingly, susceptibility to isolates of *P. tritici-repentis* that produce these toxins, have been mapped to their resident chromosomes in wheat. *Tsn1* mapped to the long arm of the 5B chromosome, *Tsc1* mapped to the short arm of the 1A chromosome, and *Tsc2* mapped to the short arm of chromosome 2B. That independent loci control the response of wheat to each HST was confirmed by their residence on different chromosomes.

Interestingly, *Tsn1* was first identified as a recessive insensitivity and resistance locus to Ptr ToxA and isolates that produce it, respectively. As such, it was designated *tsn1* (Faris et al. 1996). However, substitution of the 5B chromosome from a sensitive wheat genotype into an insensitive 5BL deletion line conferred sensitivity to Ptr ToxA, revealing that insensitivity is not conditioned by a gene product per se, but rather by the absence of the gene for Ptr ToxA sensitivity (Anderson et al. 1999). Efforts to clone *Tsn1* are ongoing (Haen et al. 2004, Lu and Faris 2006, Lu et al. 2006) and its identification promises to further our knowledge of the *P. tritici-repentis*-wheat interaction. Although all major tan spot genes from wheat thus far identified are racespecific, race-nonspecific resistance was recently identified (Faris and Friesen 2005). QTLs on the short arm of chromosome 1B and the long arm of chromosome 3B were significantly associated with resistance to races 1, 2, 3, and 5, irrespective of which HSTs
these races produce. A combination of race-specific and race-nonspecific resistance should prove beneficial for control of tan spot of wheat.

The *Pyrenophora tritici-repentis*—wheat pathosystem: a model for inverse gene-for-gene

In classical gene-for-gene interactions, the initiation of defense responses is governed by the expression of a dominant avirulence gene in the pathogen and a corresponding dominant resistance gene in the host (Bonas and Lahaye 2002, Dangl and Jones 2001, Flor 1971). Host defense initiated in this manner is often associated with the hypersensitive response, a host-mediated localized cell death that may serve to marginalize the pathogen before it invades (reviewed by Heath 2000). Due to their involvement in the elicitation of resistance, avirulence gene products have been termed agents of incompatibility (Wolpert et al. 2002). Evidence is emerging that the interaction of some HST-producing pathogens with their hosts also follows a gene-for-gene model where toxin production by the pathogen and toxin sensitivity in the host are each conferred by single dominant genes, however the outcome is instead susceptibility (Lamari et al. 2003, Strelkov and Lamari 2003, Walton 1996, Wolpert et al. 2002). As such, HSTs have been referred to as agents of compatibility (Walton 1996) and the interactions they mediate described as inverse gene-for-gene interactions (Wolpert et al. 2002). Evidence that three dominant, independently inherited genes in susceptible wheat control sensitivity to each separate HST produced by *P. tritici-repentis*, either Ptr ToxA, Ptr ToxB, or Ptr ToxC, positions the *P. tritici-repentis*—wheat pathosystem as a model system to study inverse gene-for-gene as a basis for disease specificity (Lamari et al. 2003, Strelkov and Lamari 2003, Wolpert et al. 2002).

Variability in *Pyrenophora tritici-repentis*

An understanding of *P. tritici-repentis* variation is required for effective management of tan spot of wheat (De Wolf et al. 1998). The most obvious differences
between isolates of *P. tritici-repentis* is the differential presence of various HSTs, although additional layers of diversity have also been identified. A comparison of pathogenic *P. tritici-repentis* isolates that produce Ptr ToxA with nonpathogenic isolates that lack toxin production revealed substantial differences besides just the presence and absence, respectively, of the ToxA gene and Ptr ToxA production (Lichter et al. 2002). These differences include major karyotype polymorphisms and the absence of fungal transposable elements and peptide synthetase sequences in the nonpathogenic isolates as compared to the pathogenic isolates. However, similarities were also detected, most notably that the genetic material of the chromosome harboring ToxA is found within a smaller chromosome of the nonpathogenic isolate.

Considerable variability between isolates of *P. tritici-repentis* has also been revealed by population genetic analyses. Singh and Hughes (2006) and dos Santos and colleagues (2002) used random amplified polymorphic DNA (RAPD) analysis to investigate populations of *P. tritici-repentis* in Brazil and western Canada, respectively. The considerable intraspecific genetic diversity detected was found to be independent of geography (dos Santos et al. 2002, Singh and Hughes 2006) and race (Singh and Hughes 2006). Results of an amplified fragment length polymorphism (AFLP) analysis of a global collection of *P. tritici-repentis* isolates corroborated the considerable genetic diversity of *P. tritici-repentis* isolates and its lack of correlation with race or geography (Friesen et al. 2005) as suggested by RAPD analysis. The diversity detected is surprising considering that *P. tritici-repentis* is homothallic, or self-fertile. Singh and Hughes (2006) attribute the observed genetic diversity to sexual recombination, which is feasible if the multinucleate cells of *P. tritici-repentis* are heterokaryotic. Friesen and colleagues (2005) attribute it to outcrossing, which has also been evoked to explain the diversity displayed by a globally unified population of another plant-pathogenic homothallic ascomycete, *Gibberella zeae* (Zeller et al. 2004). Alternatively, we have observed the presence of anastomosis bridges between the cells of asexual conidia of *P. tritici-repentis* (data not shown) and hypothesize that nuclei transfer through these bridges could also
potentially contribute to the diversity of this fungus. However, their role in *P. tritici-repentis* biology remains to be investigated.

Regardless of the origin of *P. tritici-repentis* variability, detected differences suggest that evolution of HST production in *P. tritici-repentis* is most likely due to more than a single molecular event, i.e. addition or loss of one toxin gene (Lichter et al. 2002). If HST production had evolved by single events, isolates of *P. tritici-repentis* would be expected to be more homogeneous. Thus, the capability to transform a nonpathogen to a pathogen with a single gene, such as *ToxA*, seems to reflect the pathogenic potential of the isolate and the gene, rather than implying strict genetic relatedness as a requirement.

**Ptr ToxA: site and mode of action**

The identification of a number of potential functional sites in Ptr ToxA, including putative sites of myristoylation (Ciuffetti et al. 1997), phosphorylation (Ciuffetti et al. 1997, Zhang et al. 1997), and cell attachment (Ciuffetti et al. 1997, Meinhardt et al. 2002, Zhang et al. 1997), were used to direct functional analyses of Ptr ToxA. Of particular interest was the identification of an Arg-Gly-Asp (RGD)-containing cell attachment motif 10 aa in length with 60% identity (Manning et al. 2006) to the RGD loop of vitronectin. Vitronectin is a protein present in the extracellular matrix of animals that relays environmental signals to cells via RGD-mediated interactions with integrin receptors located on plasma membranes (Suzuki et al. 1985). Although no integrin homologs have been identified in plants, proteins immunologically-related to integrins exist (Baluska et al. 2003, Faik et al. 1998, Kiba et al. 1998, Laboure et al. 1999, Laval et al. 1999, Nagpal and Quatrano 1999, Sun et al. 2000, Swatzell et al. 1999) and evidence suggests that RGD/integrin-like interactions occur in plants (Canut et al. 1998, Mellersh and Heath 2001). The simplest model for HST action predicts that toxin perception by the host is mediated by a host receptor (Scheffer and Livingston 1984), thus, interaction of Ptr ToxA with its host cells via the RGD cell attachment motif was the first step in the investigation of Ptr ToxA function.
The role of the RGD cell attachment motif in Ptr ToxA activity has independently been investigated by two groups, both of which found that it is important for the full activity of Ptr ToxA (Manning et al. 2004, Meinhardt et al. 2002). Meinhardt and colleagues (2002) proposed that Ptr ToxA interacts with a putative host receptor through its RGD cell attachment motif based on the combined results of site-directed mutagenesis and peptide competition assays. Ptr ToxA with RGD mutated to RAD or RGE lost the ability to cause necrosis on sensitive wheat, whereas a control mutation retained full activity. Additionally, coapplication of Ptr ToxA with an RGD tripeptide significantly inhibited toxin-induced electrolyte leakage. Via a combination of site-directed mutagenesis, peptide inhibition, and mutant protein competition analyses, Manning and colleagues (2004) revealed that, in addition to the RGD cell attachment motif, multiple motifs are necessary for complete activity of Ptr ToxA. Visualized as a loss or reduction of activity upon infiltration of mutant proteins into sensitive wheat, site-directed mutagenesis of the entire vitronectin-like motif of Ptr ToxA revealed that 9 of the 10 residues of the vitronectin-like region of Ptr ToxA, including the RGD residues, as well as two consensus phosphorylation sites predicted to be phosphorylated by casein kinase II, are required for Ptr ToxA function. Inhibition of Ptr ToxA activity by the sequence-specific peptide RGDV and a fusion protein with RGD mutated to RAD corroborated results of site-directed mutagenesis. However, a lack of structural information precluded the conclusive deduction as to which residues were important for structural reasons and which might directly be involved in Ptr ToxA function.

Solution of a high-resolution crystal structure of Ptr ToxA allowed the correlation of structure with the results of site-directed mutagenesis (Sarma et al. 2005). Mapping of existing mutation data onto the structure supports the hypothesized importance of the RGD cell attachment motif and its surrounding sequence. The occurrence of the vitronectin-like motif, including RGD, on a solvent-exposed loop in the protein suggests that it is directly involved in recognition events required for Ptr ToxA action. Additionally, Ptr ToxA was shown to be related to a classic mammalian RGD-containing domain, the fibronectin type III (FnIII) domain, via circular permutation. The similar
topology and the positional conservation of the RGD-containing loop between Ptr ToxA and mammalian FnIII proteins provides a compelling argument for common ancestry of the proteins and supports the hypothesis that Ptr ToxA binds an integrin-like receptor to gain entry to the plant host. However, confirmation of this conclusion awaits isolation and characterization of the Ptr ToxA receptor.

In an effort to further functionally characterize Ptr ToxA, Manning and Ciuffetti (2005) investigated where Ptr ToxA exerts its effects in its target cell. Contrary to the expectation that Ptr ToxA acts extracellularly, Ptr ToxA was shown to independently cross plant cell plasma membranes in the absence of the pathogen and localize to the cytoplasm and chloroplasts of sensitive, but not insensitive, wheat mesophyll cells. Evidence of Ptr ToxA internalization into toxin-sensitive cells includes: 1. protection of Ptr ToxA from protease degradation in the leaf apoplast; 2. the intracellular detection of Ptr ToxA by immunolocalization; and, 3. the direct visualization via fluorescence and confocal microscopy of functional green fluorescent protein-tagged Ptr ToxA in the cytoplasm and in association with chloroplasts. Although Ptr ToxA was only internalized into sensitive cells, internal expression of Ptr ToxA in both sensitive and insensitive wheat cells results in cell death as demonstrated by biolistic bombardment. This suggests that all of the requirements for cell death induction are present in both sensitive and insensitive cells once Ptr ToxA gains entry. Thus, the difference between insensitive and sensitive cells seems to be the recognition of Ptr ToxA at the cell membrane and subsequent import. Additionally, since both sensitive and insensitive cells have the ability to respond to Ptr ToxA once it is inside the cell, it is likely that the product of the gene controlling sensitivity to Ptr ToxA, Tsn1, is responsible for Ptr ToxA internalization. It is known that the interaction in animal cells of some RGD-containing proteins with integrins results in the internalization of the receptor and its ligands (Hynes 2002, Marjomaki et al. 2002, Memmo and McKeown-Longo 1998, Wickham et al. 1993, Zubieta et al. 2005), thus it is plausible that Ptr ToxA internalization relies on recognition of the RGD cell attachment motif by a plant protein receptor.
The localization of Ptr ToxA to chloroplasts following internalization suggests the chloroplast is a possible site of action for Ptr ToxA (Manning and Ciuffetti 2005). Indeed it has been shown that thylakoid structure is disrupted in Ptr ToxA-treated, Ptr ToxA-sensitive wheat (Freeman et al. 1995) and that Ptr ToxA activity is light dependent (Manning and Ciuffetti 2005). In further support of the chloroplast as the site of Ptr ToxA action, Ptr ToxA has also been shown to interact with a chloroplast-localized protein, designated ToxA binding protein 1 (ToxABP1) (Manning et al. 2006). ToxABP1 was identified as a binding partner for Ptr ToxA via a screen of a yeast-2-hybrid library constructed from Ptr ToxA-sensitive wheat leaves. Western analysis with an antibody to the ToxABP1 homolog from Arabidopsis, Thfl, confirmed that ToxABP1 in sensitive chloroplast extracts interacts with immobilized Ptr ToxA. The same form of ToxABP1 is expressed in both sensitive and insensitive wheat, as well as in a 5BL wheat deletion line lacking the Ptr ToxA sensitivity locus, Tsn1. This is consistent with internalization mediating the specificity of the interaction between P. tritici-repentis and wheat, rather than it being mediated at the site of Ptr ToxA action. The identification of ToxABP1 homologs in cyanobacteria and plants has provided insight into how ToxABP1 may mediate Ptr ToxA-induced necrosis, which is the focus of current investigation in our laboratory.

**Ptr ToxB: evolutionary considerations**

Unlike most other HSTs, the genetic determinants of which are present only in HST-producing, pathogenic isolates and are completely absent in nonpathogenic counterparts (Conners 1937, Kimura et al. 1998, Pitkin et al. 1996, Tanaka et al. 1999, Yang et al. 1996, Yoder et al. 1997), ToxB homologs are found in races that do not produce Ptr ToxB, nor are pathogenic on Ptr ToxB-sensitive wheat. Wild-type ToxB was originally identified in Ptr ToxB-producing race 5 isolates of P. tritici-repentis, homologs of which were subsequently identified in race 3 isolates, which are presumed to produce Ptr ToxC and are pathogenic on Ptr ToxC-sensitive wheat, and race 4 isolates, which are nonpathogenic on all wheat cultivars thus far tested. The sequence of the ToxB homolog
in race 3 is nearly identical to the wild-type ToxB open reading frame (ORF) except for the first six nucleotides; however its upstream sequence is unique and potentially contains an alternate translational start site (Strelkov et al. 2006). The ToxB homologs thus far identified in nonpathogenic *P. tritici-repentis* race 4 isolates are 86% identical to wild-type ToxB and have one additional codon in their nucleotide sequences, as well as unique flanking sequences as compared to wild-type ToxB (Martinez et al. 2004, Strelkov et al. 2006). Additionally, a comparison of the upstream sequences of the ToxB homologs in races 3 and 4 reveals sequences distinct from one another (data not shown).

Despite these upstream sequence differences between the ToxB homologs in races 3 and 4 as compared to those upstream of wild-type ToxB in race 5, all three loci are expressed in conidia as shown by RT-PCR (Strelkov et al. 2006). Wild-type ToxB in race 5 is also uniquely expressed in mycelia, except for the wildtype ToxB loci in low-virulent race 5 isolate 92-171R5 from Canada (Strelkov et al. 2006). The wild-type ToxB loci in race 5 isolate 92-171R5 share an expression pattern with the ToxB homologs in races 3 and 4 in that they are expressed only in conidia (Strelkov et al. 2006). This differential expression between wild-type ToxB loci found in low and high virulence race 5 isolates also is attributed to distinct upstream sequences (Strelkov et al. 2006), although it is unknown if the sequences upstream of the wild-type ToxB sequences in 92-171R5 are similar to those upstream of either ToxB homolog in race 3 or race 4. Two geographically distinct isolates, one from Algeria, Alg 3-24 and one from North Dakota, DW7, share at least three identical ToxB loci, including both the ORF and flanking sequences. Thus, the discovery of a North American race 5 isolate with a ToxB expression pattern similar to that of distinct ToxB homologs in nonpathogenic *P. tritici-repentis* race 3 and 4 isolates is curious. It is unclear why ToxB from a North Dakota race 5 isolate is more similar to a North African race 5 isolate than to a race 5 isolate from Canada (Strelkov et al. 2006). Nevertheless, flanking sequence variability and expression differences for wild-type ToxB loci in various race 5 isolates, and the presence of active ToxB homologs in various races of *P. tritici-repentis* that are not pathogenic on
Ptr ToxB-sensitive wheat, suggests a complex evolutionary history for this HST in *P. tritici-repentis*.

**Overview of Dissertation**

The overall objective of this research is to further our understanding of the molecular basis of the disease interaction between *P. tritici-repentis* and wheat. In chapter 2, the genetic diversity of *P. tritici-repentis* is further revealed, in that two new races were identified via a combination of phenotypic and genotypic analyses. Further insight into the evolution of ToxB is provided in chapters 3 and 4. In chapter 3, the distribution of ToxB-like sequences is shown to extend beyond the races of *P. tritici-repentis* to other fungal relatives. In chapter 4, the function of one ToxB homolog in another plant pathosystem is investigated. Finally, fluorescent tools for use in fungal-host interactions and fungal biology are presented in chapter 5.
Chapter 2

A combination of phenotypic and genotypic characterization reveals two new races of *Pyrenophora tritici-repentis*

Rachael M. Andrie, Iovanna Pandelova, and Lynda M. Ciuffetti

Prepared for Submission
Abstract

*Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, produces multiple host-selective toxins (HSTs), including Ptr ToxA, Ptr ToxB, and Ptr ToxC. The specific complement of HSTs produced by a particular isolate determines its host cultivar specificity. Each unique specificity profile, visualized as the differential induction of necrosis and/or chlorosis on a standard set of wheat differentials, defines a unique race. Eight races of *P. tritici-repentis* have been formally published, although additional races are under investigation. Despite its obvious utility, we found the use of disease phenotype alone for race classification can be misleading. Inoculation of the *P. tritici-repentis* isolates SO3 and PT82 on the current wheat differential set indicated classification as race 2 and race 8, respectively; however, genetic characterization revealed these isolates do not possess all of the associated HSTs expected for these race assignments. Despite sharing disease phenotypes similar to known races, SO3 and PT82 were genotypically distinct from these previously characterized races of *P. tritici-repentis*. Thus, a combination of genotypic and phenotypic characterization established two new races, which we designated as race 9 (SO3) and race 10 (PT82). To ensure detection of the breadth of physiological variation present among the isolates of *P. tritici-repentis*, our results indicate that race classification should include both phenotypic and genotypic analyses, and eventual expansion of the differential set.

Introduction

*Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, is an economically important pathogen of wheat worldwide (Ciuffetti and Tuori 1999, De Wolf et al. 1998, Strelkov and Lamari 2003). Methods used to categorize isolates of *P. tritici-repentis* have evolved in step with our emerging understanding of the complexity of the interaction between *P. tritici-repentis* and wheat. Prior to the identification of the toxic components produced by *P. tritici-repentis*, Lamari and Bernier (1989a) developed an isolate categorization system based on lesion type. Isolates were arranged into four pathotypes based on the ability to produce necrosis and/or chlorosis on wheat. Pathotype
isolates produce both necrosis and chlorosis; isolates of pathotype 2 and 3 produce only one symptom each, necrosis or chlorosis, respectively; and, nonpathogenic pathotype 4 isolates lack either symptom. Of the wheat differentials used to create the pathotype system, the cultivar Glenlea and the line 6B365 came into consistent use for the detection of necrosis and chlorosis, respectively, when designating an isolate to pathotype (De Wolf et al. 1998). It is now known that necrosis on Glenlea can be attributed to production of Ptr ToxA (Synonyms: Ptr necrosis toxin, Ptr toxin and ToxA [Ciuffetti et al. 1998]) (Ballance et al. 1989, Tomas et al. 1990, Tuori et al. 1995, Zhang et al. 1997), a 13.2 kDa (Tuori et al. 1995) proteinaceous host-selective toxin (HST) encoded for by ToxA (Ballance et al. 1996, Ciuffetti et al. 1997), and that chlorosis on 6B365 can be attributed to production of Ptr ToxC (Effertz et al. 2002), which appears to be a nonionic, polar, low molecular weight HST, the gene(s) for which has yet to be characterized.

The discovery of a distinct chlorotic symptom on the wheat cultivar Katepwa caused by a previously uncharacterized isolate of P. tritici-repentis led to refinement of the pathotype system (Lamari et al. 1995). Lamari and colleagues (1995) proposed a race designation system modeled after that of cereal rusts to describe the isolates of P. tritici-repentis. The new race-based categorization system is limited only by the size and effectiveness of the differential set, in contrast to the original pathotype classification system that allowed the description of only four pathotypes. The four pathotypes previously described became races 1 through 4, and a new isolate was designated as race 5. Additionally, the wheat line 6B662, formerly considered resistant to P. tritici-repentis, was introduced as the differential for race 5. Chlorosis on Katepwa and 6B662 is now known to be due to the production of Ptr ToxB (Synonym: Ptr chlorosis toxin [Ciuffetti et al. 1998]), a 6.6 kDa proteinaceous HST (Orolaza et al. 1995, Strelkov et al. 1998, Strelkov et al. 1999) encoded for by ToxB (Martinez et al. 2004, Martinez et al. 2001). Distinct ToxB-like sequences have been identified in isolates of race 3 (Strelkov and Lamari 2003) and 4 (Martinez et al. 2004, Strelkov and Lamari 2003), although neither race produces Ptr ToxB (Ciuffetti et al. 2003, Strelkov and Lamari 2003) nor is pathogenic on Ptr ToxB-sensitive wheat (Lamari et al. 1995). In race 3, all but the first
six nucleotides of the ToxB-like sequence are identical to ToxB (Strelkov et al. 2006); whereas, for race 4, the sequences are more divergent (Martinez et al. 2004, Strelkov et al. 2006). For example, toxb from the race 4 isolate SD20 shares 86% identity with ToxB (Martinez et al. 2004).

The virulence patterns of the various races of *P. tritici-repentis* have since been discovered to be toxin-based in that each compatible interaction between an isolate and its corresponding susceptible host differential is mediated by a HST (Strelkov and Lamari 2003). The specific complement of HSTs produced by a particular isolate therefore determines its race. It has been shown that Ptr ToxA causes necrosis on Glenlea and Katepwa; Ptr ToxB causes chlorosis on 6B662 and Katepwa; and Ptr ToxC causes chlorosis on 6B365. Isolates producing all possible combinations of these three HSTs have been identified and are accordingly categorized as races 1 to 8 (Lamari et al. 1995, Lamari et al. 2003, Strelkov et al. 2002). Table 2.1 summarizes the race structure of *P. tritici-repentis* on the commonly-used wheat differential set (Lamari and Bernier 1989a, Lamari et al. 1995, Strelkov and Lamari 2003) and indicates the toxin production profiles of each given race. The wheat cultivars Salamouni (De Wolf et al. 1998, Strelkov and Lamari 2003) and Auburn (Ciuffetti et al. 1997, Tomas and Bockus 1987, Tomas et al. 1990, Tuori et al. 1995) are resistant to all known races of *P. tritici-repentis* and insensitive to its known toxins.

An understanding of *P. tritici-repentis* physiological variation is required for effective management of tan spot of wheat (De Wolf et al 1998). Despite its obvious utility in revealing such variation, we found that race classification of *P. tritici-repentis* isolates based solely on phenotype has the potential to be misleading. Here we report two examples where genetic characterization differed from phenotypically-determined race designations. Although *P. tritici-repentis* isolate SO3 is known to lack ToxA (Ciuffetti et al. 1997), it unexpectedly produced a Ptr ToxA-associated disease phenotype upon inoculation. Additionally, disease phenotype alone led us to mistakenly classify an isolate from Kansas (designated PT82) as race 8 (Andrie et al. 2003), which is indicated
### Table 2.1. Race structure of *Pyrenophora tritici-repentis*.\(^a\)

<table>
<thead>
<tr>
<th>Race</th>
<th>Glenlea</th>
<th>Katepwa</th>
<th>6B662</th>
<th>6B365</th>
<th>Sal(^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N (ToxA)</td>
<td>N (ToxA)</td>
<td>R</td>
<td>C (ToxC)</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>N (ToxA)</td>
<td>N (ToxA)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>R(^g)</td>
<td>R(^g)</td>
<td>C (ToxC)</td>
<td>R</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R(^g)</td>
<td>R(^g)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>C (ToxB)</td>
<td>C (ToxB)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>6</td>
<td>R</td>
<td>C (ToxB)</td>
<td>C (ToxB)</td>
<td>C (ToxC)</td>
<td>R</td>
</tr>
<tr>
<td>7</td>
<td>N (ToxA)</td>
<td>N (ToxA)</td>
<td>C (ToxB)</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>8</td>
<td>N (ToxA)</td>
<td>N (ToxA)</td>
<td>C (ToxB)</td>
<td>C (ToxC)</td>
<td>R</td>
</tr>
</tbody>
</table>

\(^a\)Based on references: Lamari and Bernier 1989, Strelkov et al. 2002, and Lamari et al. 2003

\(^b\)N = necrosis; C = chlorosis; R = resistance

\(^c\)ToxA = presence of *ToxA* and production of Ptr ToxA

\(^d\)ToxB = presence of *ToxB* and production of Ptr ToxB

\(^e\)ToxC = production of Ptr ToxC as evidenced by inoculation

\(^f\)Sal = Salamouni

\(^g\)Although race 3 (Strelkov et al. 2003) and race 4 (Martinez et al. 2004, Strelkov et al. 2006) possess distinct *ToxB* homologs, neither race produces Ptr ToxB (Ciuffetti et al. 2003, Strelkov et al. 2006) nor is pathogenic onPtr ToxB-sensitive wheat (Lamari and Bernier 1989; Lamari et al. 1995).
by the production of the three characterized HSTs, Ptr ToxA, Ptr ToxB and Ptr ToxC, although genotypic analysis revealed it does not possess all of the associated HSTs expected for this race assignment. Based on these observed discrepancies, our objective was to demonstrate that race classification based on the current set of published differentials should include both phenotypic and genotypic characterization. The combination of these analyses revealed two new races of *P. tritici-repentis* that apparently produce phytotoxic activities distinct from Ptr ToxA, Ptr ToxB, and Ptr ToxC.

**Materials and methods**

**Fungal isolates**

The following two *P. tritici-repentis* isolates were chosen for race characterization: SO3, collected from a field in southern Oregon (M. L. Putnam and L. M. Ciuffetti, unpublished data) and originally designated EO3 (Ciuffetti et al. 1997), and PT82, collected from a field in Kansas. For comparison, the following *P. tritici-repentis* isolates served as representatives of races 1 through 5: for race 1, isolate SD8; for race 2, isolate 86-124 (Lamari and Bernier 1989b, Lamari et al. 1995); for race 3, isolate D308 (Lamari and Bernier 1989b, Lamari et al. 1995); for race 4, isolate SD20 (Martinez et al. 2004); and, for race 5, isolate DW7 (Ali et al. 1999). We were unable to obtain isolates of the published races 6 (Strelkov et al. 2002), 7 and 8 (Lamari et al. 2003), thus information presented here on these races is based on published data.

**Culture conditions**

Cultures of *P. tritici-repentis* were grown on solid V8 juice agar (20% v/v V8 juice, 0.3% w/v CaCO₃, 2% w/v Bacto agar) in constant darkness, 25°C. Sporulation of *P. tritici-repentis* was induced by flooding a culture with sterile water and manually depressing aerial hyphae with a glass rod, followed by incubations of 18-24 h of light at room temperature and 18-24 h of darkness at 16°C (Lamari and Bernier 1989a). To obtain mycelia for DNA extraction, *P. tritici-repentis* conidia were collected in water + 0.015% Tween-20, inoculated into 75 ml quarter-strength potato dextrose broth (BD
Difco, Sparks, MD), and grown at room temperature, 175 rpm, 16 h. Mycelia were collected and lyophilized. To obtain crude culture filtrates (CCF) for protein precipitations and toxin identification, three #3 borer plugs of V8 agar-grown mycelia were inoculated into 50 ml modified Fries medium (Tomas et al. 1990), followed by incubation as stationary cultures at 25°C under constant fluorescent light for 19 days.

Plant material and inoculations

The chosen set of wheat differentials included the cultivars Glenlea (De Wolf et al. 1998), Katepwa (Lamari et al. 1995) and Auburn (Ciuffetti et al. 1997, Tomas and Bockus 1987, Tomas et al. 1990), and the lines 6B662 (Lamari et al. 1995) and 6B365 (De Wolf et al. 1998). Prior to inoculation, plants were grown for two to three weeks under diurnal conditions of light for 16 h at 23°C and darkness for 8 h at 19°C. Differentials were inoculated with a conidial suspension from each isolate (3×10³ conidia/ml) to drip stage, incubated overnight in a darkness dew chamber at ~20°C to 22°C, then returned to diurnal conditions. Disease development was subsequently monitored and secondary leaves harvested 5 to 8 days post-inoculation to document infection phenotype. Inoculations were repeated with similar results at least four times for SO3 and PT82, and at least two times for known races 1 to 5.

Southern analyses

DNA was purified using the procedure of Martinez and colleagues (2004) via organic extraction in conjunction with a modified polysaccharide precipitation method (Michaels et al. 1994). DNA concentrations were determined via comparison to λ DNA standards on an ethidium bromide-stained 1% agarose gel. Approximately 1 µg of restriction enzyme-digested genomic DNA was size-fractionated followed by downward transfer onto membranes via alkaline transfer. For ToxA-probed Southern blots, DNA was digested with either SacI or EcoRV and transferred to GeneScreen Plus Hybridization Transfer Membranes (PerkinElmer Life and Analytical Sciences, Boston, MA); for ToxB-probed Southern blots, DNA was digested with either HindIII or EcoRV
and transferred to Zeta-Probe Blotting Membranes (Bio-Rad Laboratories, Hercules, CA). Blots were dried at 80°C for 2 h, prehybridized in 7% SDS, 0.5 M NaHPO₄, 0.001 M EDTA [pH 8.0], 1% bovine serum albumin fraction V at 60°C for at least 2 h, and hybridized with labeled probe at 60°C overnight.

Membranes were probed with either a 591 bp fragment of ToxA or a 243 bp fragment of ToxB obtained with polymerase chain reaction (PCR). Primers TA4bF (5'-GGTCATGCGTTCTATCCTCGTAC-3') and TA14bR (Tuori et al. 2000) were used to amplify the ToxA probe. The ToxB probe was amplified with primers TB57F (5'-GAGACTGCTATGCTACTTGCTG-3') and TB6R (Table 2.2) (Martinez et al. 2001, Martinez et al. 2004). The reaction mixture was composed of 1× Taq polymerase buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0), 200 μM each dNTP, 200 nM each primer, 2.5 U Taq polymerase (MBI Fermentas, Hanover, MD), and 10 ng template DNA in 50 μl. Amplification was carried out as follows: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 58°C for 30s, 72°C for 30s; 1 cycle of 72°C for 7 min. PCR products were purified on MinElute Spin Columns (Qiagen, Valencia, CA) and α-P³²-dCTP-labeled with the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA) per manufacturer's instructions.

Hybridization was followed by three washes with 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate), 0.5% SDS at 60°C for 5, 10 and 30 min, and a final high stringency wash with 0.2× SSC (0.03 M NaCl, 0.003 M trisodium citrate), 2% SDS at 60°C for 45 min. To detect probe hybridization, blots were exposed to autoradiographic film (Denville Scientific, Metuchen, NJ). At least two independent DNA samples for each isolate were analyzed via Southern analysis.

**Western analyses**

For Ptr ToxA and Ptr ToxB western analyses, total proteins were precipitated from 500 μl and 1 ml aliquots of CCF, respectively, via the trichloroacetic acid (TCA) procedure of Ciuffetti and colleagues (1997). Protein samples were fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of
Table 2.2. Multiplex PCR primers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToxA</td>
<td>TA51F</td>
<td>5'GCGTTCTATCCCTCGTACTTC-3'</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>TA52R</td>
<td>5'GCATTCTCCAATTTCACG-3'</td>
<td>This study</td>
</tr>
<tr>
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<td></td>
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<td>5'ACGTCCTCCACTTGCACTCCTC-3'</td>
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<td>CHS-354R</td>
<td>5'TGGAAGAACCATCTCTGTGAGGTTG-3'</td>
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aMartinez et al. 2001  
bMartinez et al. 2004  
cCarbone and Kohn 1999
Fling and Gregerson (1986). SDS-PAGE gels were then equilibrated in transfer buffer (25 mM Tris Base, 193 mM glycine, 20% methanol), followed by dry transfer of proteins to GE Pure Nitrocellulose Transfer Membrane (GE Osmonics, Minnetonka, MN). Resultant blots were blocked overnight at 4°C in 1x TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCL, pH 8.0), 3% nonfat dried milk. Blots were then washed in 1x TBST, followed by incubation for 1 h in either anti-Ptr ToxA N+C fusion protein antisera (Manning and Ciuffetti 2005) or anti-Ptr ToxB antisera (Andrie et al. 2005) diluted 1:10,000 or 1:2,000, respectively, in 1x TBST, 1% BSA fraction V. Blots were again washed, followed by incubation for 0.5 h in anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:8,000 in 1x TBST. Following a final wash, blots were developed via chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) per manufacturer's instructions. Western analyses were repeated on two independent CCF samples for both Ptr ToxA and Ptr ToxB.

Multiplex PCR

A modification of the FastDNA Kit (Qbiogene, Carlsbad, CA) manufacturer's instructions was used to extract genomic DNA. From 5 to 50 mg of lyophilized fungal tissue were added to lysis matrix A and homogenized 10 s at a setting of 4.5 with a FastPrep Instrument (Qbiogene, Carlsbad, CA). Immediately following homogenization, 1 ml CLS-VF cell lysis solution and 200 µl protein precipitation solution (PPS) were added to each sample, followed by a 1 min incubation and centrifugation at 14,000 x g, 10 min. The supernatant was then added to 600 µl binding matrix, incubated for 5 min, and pulsed for 5 s. The resultant pellet was gently resuspended in 500 µl SEWS-M wash solution and placed on a SPIN column (Qbiogene, Carlsbad, CA) for purification of genomic DNA. Bound DNA was eluted with 100 µl DES and quantified via DNA gel electrophoresis as described above.

Primers used for multiplex PCR are listed in Table 2.2. Primers specific to the chitin synthase 1 gene, CHS-1 (Carbone and Kohn 1999), were added as an internal
control for the presence of fungal DNA. When used with primer TB71F, primer TB60R (Martinez et al. 2004) amplifies both ToxB in race 5 and the ToxB-like sequence in race 3, whereas primer TB58R amplifies only toxb from race 4. Reaction conditions were as follows: 1× Taq polymerase buffer, 200 μM each dNTP, 200 nM each primer; 2% (v:v) DMSO, 1.25 U Taq polymerase, and 10 ng DNA template in 25 μl. Following an initial 1 min, 94°C denaturation, samples underwent 30 cycles of 94°C for 45 s, 58°C for 30s, and 72°C for 1 min, 30 s, ending with a final 7 min extension at 72°C. We repeated multiplex PCR on at least two independent DNA samples.

Results

Phenotypic characterization

The standard set of five wheat differentials were inoculated with the P. tritici-repentis isolates SO3 and PT82 to determine their race designation and thus the likely complement of HSTs they produce (Figure 2.1). The disease profiles obtained were first compared with inoculation results from known races in our possession, races 1 to 5 (Table 2.1). For races 6, 7, and 8 (see materials and methods), we relied on inoculation of races 2, 3, and 5 to generate disease phenotype standards on corresponding susceptible differentials. To date, these races have been shown to produce only one toxin each, Ptr ToxA, Ptr ToxC, or Ptr ToxB, respectively (Table 2.1). Disease phenotypes resulting from inoculation with these races were assumed to be representative for each respective toxin. Disease phenotype standards were then used to reconstruct disease profiles for races 6, 7, and 8 (Table 2.1) to compare to inoculation results for SO3 and PT82. For example, Figure 2.1B shows a disease profile reconstruction for race 8.

Even though it lacks the ToxA gene (Ciuffetti et al. 1997), the disease phenotype resulting from SO3 inoculation matched that of race 2 as demonstrated by the presence of necrotic lesions with chlorotic halos on Glenlea and Katepwa, and a resistance reaction on other tested differentials (Figure 2.1A). The disease phenotype of PT82 did not match any of the disease profiles for races 1 to 5 (data not shown). However, comparison to
Figure 2.1. Race designation of *Pyrenophora tritici-repentis* isolates SO3 and PT82 based on phenotypic characterization.

A) The disease reaction of SO3 on the current set of wheat differentials matches that of race 2 (i.e., Ptr ToxA-induced necrosis on the wheat cultivars Glenlea and Katepwa). B) PT82 produces a race 8 disease profile (i.e., Ptr ToxA-induced necrosis on Glenlea and Katepwa, Ptr ToxB-induced chlorosis on Katepwa and 6B662, and Ptr ToxC-induced chlorosis on 6B365) on the current differential set as determined by comparison to disease phenotype standards. Disease phenotype standards represent the disease reaction caused by the production of individual HSTs on corresponding sensitive differentials. These were obtained via inoculation of races that have thus far been shown to produce only one toxin each, Ptr ToxA (race 2), Ptr ToxB (race 5), or Ptr ToxC (race 3). Resultant disease phenotypes on corresponding sensitive differentials are assumed to be due to each respective toxin. Res = resistant; 2/ToxA = Race 2/Ptr ToxA; 5/ToxB = Race 5/Ptr ToxB; 3/ToxC = Race 3/Ptr ToxC; Aub = Auburn; Glen = Glenlea; Kat = Katepwa.
toxin phenotype standards revealed that PT82 shares the Ptr ToxA necrosis phenotype on Glenlea and Katepwa with race 2, the Ptr ToxC chlorosis phenotype on 6B365 with race 3, and the Ptr ToxB chlorosis phenotype on Katepwa and 6B662 with race 5 (Figure 2.1B). That PT82 exhibited all three toxin phenotypes led us to initially categorize it as race 8, which is characterized by the production of these three HSTs (Table 2.1).

Genotypic characterization

We utilized a molecular approach to evaluate the phenotypically-determined race designations of SO3 as race 2 and PT82 as race 8. The availability of the ToxA (Ballance et al. 1996, Ciuffetti et al. 1997) and ToxB (Martinez et al. 2001) genes and antibodies (Andrie et al. 2005, Manning and Ciuffetti 2005) to both toxins facilitated Southern and western analyses, respectively. Ptr ToxC awaits full characterization, thus tools for genotypic characterization are not yet available. Hybridization patterns in Southern analyses for races 1 to 5 were as expected. The ToxA probe hybridized to SacI-digested genomic DNA of races 1 and 2, but not to races 3, 4, or 5, whereas the ToxB probe hybridized to HindIII-digested genomic DNA of races 3, 4, and 5, but not to races 1 or 2 (Figure 2.2A). Comparable results were obtained for hybridization of either probe to EcoRV-digested genomic DNA (data not shown). As shown by western analyses, all races that contained ToxA produced Ptr ToxA; however, not all races with ToxB produced Ptr ToxB (Figure 2.2B). Although races 3 and 4 possess homologs of ToxB (Figure 2.2A), they do not produce detectable amounts of Ptr ToxB (Figure 2.2B), nor do they infect Ptr ToxB-sensitive wheat differentials (data not shown). As expected, only the race 5 isolate produced Ptr ToxB (Figure 2.2B).

For both SO3 and PT82, molecular characterization contradicted phenotypic race classification. Although SO3 demonstrated a Ptr ToxA phenotype on Glenlea and Katepwa (Figure 2.1A), and mimics a race 2 disease profile, it is known to lack the ToxA gene (Ciuffetti et al. 1997). Due to this discrepancy, we reconfirmed the absence of ToxA (Figure 2.2A) and Ptr ToxA production (Figure 2.2B), in turn confirming that SO3 is clearly not a race 2 isolate. Consistent with its designation as race 8, PT82 possessed the
Figure 2.2. Genetic characterization of SO3 and PT82 differs from phenotypically-determined race designations.

A) Southern analyses of SO3 and PT82 as compared to *Pyrenophora tritici-repentis* races 1 to 5. One microgram of *SacI*- or *HindIII*-digested genomic DNA from each isolate was hybridized with a *ToxA* or *ToxB* probe, respectively.

B) Western analyses of SO3 and PT82 as compared to *P. tritici-repentis* races 1 to 5. For each isolate, total precipitated protein from 500 µl or 1 ml crude culture filtrate was subjected to western analyses with Ptr ToxA- or Ptr ToxB-specific antibodies, respectively.
ToxA gene (Figure 2.2A) and produced Ptr ToxA (Figure 2.2B); however, it lacked the ToxB gene (Figure 2.2A) and Ptr ToxB production (Figure 2.2B), despite causing chlorosis on Katepwa and 6B662 (Figure 2.1B). Thus, new phytotoxic activities produced by SO3 and PT82 on the current wheat differential set were implicated by genotypic characterization.

Multiplex PCR

To facilitate rapid characterization of P. tritici-repentis isolates, a PCR-based screen was developed to simultaneously identify disease determinants whose genes have been characterized. Primer sets were designed to amplify portions of both the ToxA and ToxB open reading frames (Table 2.2). The ToxA primer set amplified a 573 bp fragment; whereas, the ToxB primer set amplified a 232 bp fragment (Figure 2.3A). Primers (Table 2.2) for CHS-1 (Carbone and Kohn 1999), the gene for chitin synthase, were included as an internal control for the presence of fungal DNA and produced a 275 bp amplification product (Figure 2.3). These three amplification product sizes were easily distinguished by DNA agarose gel electrophoresis and thus proved useful for multiplex PCR.

Multiplex PCR amplification patterns for races 1 to 5 were as expected based on results of Southern analyses (Figure 2.2A). Races 1 and 2 tested positive with primers specific to ToxA; races 3, 4, and 5 tested positive with primers specific to ToxB (Figure 2.3A). Two additional primer sets (Table 2.2) differentiated the ToxB sequences in races 3 and 5 from toxb, the ToxB homolog in race 4 isolate SD20 (Figure 2.3B and C). When paired with the forward primer TB71F, reverse primer TB60R (Martinez et al. 2004) was specific to the ToxB sequences in races 3 and 5 (Figure 2.3B), whereas reverse primer TB58R was specific to toxb in race 4 (Figure 2.3C). Multiplex PCR amplification patterns for SO3 and PT82 also confirmed Southern analyses (Figure 2.2A). SO3 tested negative for both genes, whereas PT82 tested positive for ToxA, but not ToxB. A CHS amplification product was detected in every PCR reaction that contained fungal DNA (Figure 2.3)
Figure 2.3. Multiplex PCR detection of host-selective toxin genes in isolates of *Pyrenophora tritici-repentis*.

A) Agarose gel electrophoresis of multiplex PCR amplification products from genomic DNA of SO3, PT82, and races 1 to 5. B) Multiplex PCR modified to include *ToxB*-specific primers. C) Multiplex PCR modified to include *toxb*-specific primers. Primers for the chitin synthase 1 gene, *CHS-1*, provide an internal control for the presence of fungal DNA.
Discussion

*P. tritici-repentis* exhibits a complex race structure in its interaction with wheat (Table 2.1). Eight races are distinguished by distinct disease reactions on current wheat differentials. The various virulence phenotypes can be attributed to the differential expression of the three currently identified HSTs produced by this fungus. Additional races of *P. tritici-repentis* have been suggested but await publication in a peer-reviewed journal (Ali and Francl 2002, Ali et al. 2002, Ciuffetti et al. 2003, Manning et al. 2002, Meinhardt et al. 2003). Isolates are typically designated as a particular race by comparison of disease phenotype profiles on a standard set of wheat differentials in comparison to that of known races, however, our results suggest genotypic characterization should be included for definitive race designation. Although phenotypic classification is necessary and informative, we present two examples where race designations and associated toxin production profiles were erroneously assigned to *P. tritici-repentis* isolates based on disease phenotype alone. Inoculation of the *P. tritici-repentis* isolates SO3 and PT82 on the standard wheat differential set indicates classification as race 2 and 8, respectively (Figure 2.1). However, genetic characterization reveals these isolates do not possess all of the associated HSTs expected for these race assignments (Figure 2.2 and 2.3), and thus SO3 is clearly not race 2, nor is PT82 race 8. A combination of genotypic and phenotypic characterization was necessary to classify these isolates as two new races of *P. tritici-repentis*, which we designate here as race 9 (SO3) and race 10 (PT82).

In the process of identifying two new races of *P. tritici-repentis*, we uncovered two new potentially toxic activities on wheat (for SO3, the necrotic disease reaction on Glenlea and Katepwa [Figure 2.1A]; for PT82, the chlorotic disease reaction on Katepwa and 6B662 [Figure 2.1B]). Official declaration of these as HSTs awaits confirmation of the criteria for standardized nomenclature, which was designed to coordinate efforts to characterize the HSTs produced by *P. tritici-repentis* (Ciuffetti et al. 1998). Criteria for naming a new HST include: 1. toxin characterization or proof that it is not one of the existing toxins; 2. demonstration of toxin specificity; and 3. demonstration of toxin
involvement in disease development. Characterization of the necrotic activity produced by SO3 on Glenlea and Katepwa, which we hypothesize to be caused by a previously undescribed HST, is currently in progress in our laboratory (Ciuffetti et al. 2003; Pandelova and Ciuffetti 2005) and has been tentatively named Ptr ToxD (Manning et al. 2002). It must be noted that an independent toxic activity was also simultaneously labeled as Ptr ToxD (Ali et al. 2002, Meinhardt et al. 2003), although official designation of either toxic activity as Ptr ToxD awaits formal publication. Just as there are examples where one name has been used to describe two distinct HSTs produced by P. tritici-repentis (Ciuffetti et al. 1998), two isolates with distinct disease profiles have been labeled as the same race (Ali and Franc! 2002, Alit et al. 2002, Lamari et al. 2003, Meinhardt et al. 2003). The tan spot community is aware of the need for standardization of the criteria required for race classification and a collaborative publication similar to that done for P. tritici-repentis HST nomenclature is anticipated.

Ideally, race classification would be based on a standard set of differentials made up of distinct cultivars specific to each unique toxic activity produced by P. tritici-repentis. Indeed, identification of a cultivar specific for the toxic activity produced by SO3 is a current effort in our laboratory, although we have yet to identify a cultivar that separates sensitivity to Ptr ToxA from sensitivity to the toxic activity produced by SO3 (Pandelova, Riera-Lizarazu, and Ciuffetti, unpublished data). Although expansion of the differential set to include cultivars specific to each new activity produced by P. tritici-repentis is a logical step toward standardization of race classification, its implementation in lieu of the inclusion of genotypic characterization deserves careful consideration. First, it is likely that the scenario described here, that two distinct toxic activities cause similar symptoms on the same host genotype, is not unique. Thus, exclusion of genetic confirmation of phenotypic race characterization could lead to similar misclassifications. Second, infinite expansion of the differential set to account for each newly characterized toxic activity could eventually make race classification based on phenotype too cumbersome for individual laboratory implementation (Caten 1987). The ease with
which genotypic characterization can be executed provides a useful complement to
disease phenotype in the classification of *P. tritici-repentis* isolates to race.

A combination of phenotypic and genotypic characterization has also been used
for race classification in *Cochliobolus carbonum*, causal agent of Northern leaf spot and
ear rot of maize (*Zea mays* L.). Five races of this pathogen have been identified,
including nonvirulent race 0 (Welz and Leonard 1993) and virulent races 1 to 4 (Dodd
of race 1, which is unequivocally identifiable by disease phenotype due to the production
of the HST HC-toxin (Gross et al. 1982, Pope et al. 1983, Walton et al. 1982), Jones and
Dunkle (1993) declared *C. carbonum* race identification based on disease reaction as
ambiguous, faulting both phenotypic variation between different isolate/cultivar
combinations and the use of an inconsistent set of inbred lines for race characterization.
As such, they examined whether fungal genotyping could be used to discern races of *C.
carbonum*. Their results indicate that PCR amplification with both arbitrary and gene-
specific primers successfully differentiates among all but two of the races of *C.
carbonum*. Races 2 and 4 of *C. carbonum* are genotypically identical despite having
distinct disease phenotypes, which is the converse to our situation with *P. tritici-repentis*.

Indeed, the versatility of PCR-based methodologies has led to its widespread
application to investigations in plant pathology (Henson and French 1993). Races of
fungal pathogens are most commonly distinguished genotypically by molecular marker
methods that employ arbitrary primers, such as random amplified polymorphic DNA
(RAPD) and amplified fragment polymorphism (AFLP) assays (Abdennadher and Mills
Woo et al. 1996, Zhong and Steffenson 2001a), although some methods utilize specific
primers (Jiménez-Gasco and Jiménez-Díaz 2003, Xue et al. 1992). As AFLP analysis did
not differentiate between the races of *P. tritici-repentis* (Friesen et al. 2005), we took
advantage of the intimate association between HST production and race identity to
develop a straight-forward and high-throughput multiplex PCR assay based on HST
gene-specific primers (Table 2.2) to aid in race differentiation (Figure 2.3).

*ToxA* and *ToxB* are the only HST genes available for use in genetic screens of *P. tritici-repentis*. Due to the world-wide distribution of *ToxA- and ToxB*-containing races of *P. tritici-repentis* (Ali and Francl 2003a, Ali et al. 2004, Engle et al. 2004, Lamari and Bernier 1989b, Lamari et al. 1998, Lamari et al. 2005, Strelkov and Lamari 2003), our multiplex PCR screen promises to be very useful for characterization of isolates to race. Even so, genotypic race characterization alone cannot yet replace phenotypic race designation. Foremost, all of the disease determinants involved in the interaction between *P. tritici-repentis* and wheat have yet to be characterized. The most glaring omission from our multiplex PCR assay is a screen for the presence of *Ptr ToxC*. The gene(s) involved in the synthesis of *Ptr ToxC*, a disease determinant produced by the *P. tritici-repentis* races 1, 3, 6, and 8, has yet to be cloned. Because race 1 isolates are the most prevalent race in the world, and race 3 isolates have been found in North America (Engle et al. 2004, Strelkov and Lamari 2003), Europe (Ali et al. 2004), and Asia (Lamari et al. 2005, Strelkov and Lamari 2003), *Ptr ToxC* is the leading candidate for inclusion in the multiplex PCR screen.

The presence of *ToxB* alleles in races that are not pathogenic on *Ptr ToxB*-sensitive wheat, in particular in race 3 (Strelkov et al. 2006) and race 4 (Martinez et al. 2004, Strelkov et al. 2006), also confounds exclusive use of our multiplex PCR to designate race. Although nonpathogenic race 4 isolates can be identified with our *toxb*-specific primers, our *ToxB*-specific primers recognize both *ToxB* in race 5 and its homolog in race 3 isolates (Figure 2.3C). Because race 3 isolates do not produce *Ptr ToxB* (Figure 2.2B), it follows that all positive multiplex PCR results obtained with our *ToxB*-specific primer set require confirmation of *Ptr ToxB* expression via western analysis (Figure 2.2B) or direct isolation of the toxin from crude culture filtrate. Even so, multiplex PCR serves to limit the number of isolates that necessitate these more laborious manipulations. Alternatively, development of a race 3-specific primer set could alleviate this concern.
Our results reveal that a combination of phenotypic and genotypic characterization can be used to designate new races of *P. tritici-repentis*. Despite the production of familiar disease phenotypes by *P. tritici-repentis* isolates SO3 and PT82, genotypic characterization unveiled the absence of the HSTs suggested by inoculation of the differential set. As such, we designated these previously unidentified phenotype/genotype combinations of SO3 and PT82 as races 9 and 10, respectively. The likely discovery of new toxic activities produced by *P. tritici-repentis*, both as described here on the current differential set and as predicted on an expanded differential set (Caten 1987, Lamari et al. 2005), suggests an even more complex race structure for *P. tritici-repentis* than already described. Additionally, in a community effort that is currently in progress in our laboratory in collaboration with the Broad Institute (Galagan et al. 2005), completion of the genomic sequence of a race 1 isolate and the production of EST libraries from additional races promises to significantly increase the discovery rate of pathogenicity/virulence factors produced by this fungus. The complexity of the interaction between *P. tritici-repentis* and wheat emphasizes the need for systematic characterization of the HSTs and races of *P. tritici-repentis*, and for the availability of all published races and differentials for accomplishing this goal.
Chapter 3

*Tox*\(\text{B}\), a host-selective toxin gene from *Pyrenophora tritici-repentis*, is present in the genome of sister-species *P. bromi* and other members of the Ascomycota

Rachael M. Andrie, Rebecca Tippner-Hedges, Joseph Spatafora, and Lynda M. Ciuffetti

Prepared for Submission
Abstract

*Pyrenophora tritici-repentis* requires the production of host-selective toxins (HSTs) to cause the disease tan spot of wheat, including Ptr ToxA, Ptr ToxB, and Ptr ToxC. *P. bromi*, the species most closely related to *P. tritici-repentis*, is the causal agent of brown leaf spot of bromegrass. Because of the relatedness of *P. bromi* and *P. tritici-repentis*, we investigated the possibility that *P. bromi* contains sequences homologous to *ToxA* and/or *ToxB*, the products of which may be involved in its interaction with bromegrass. Multiplex polymerase chain reaction (PCR) revealed the presence of *ToxB*-like sequences in *P. bromi*, which we have named *PbToxB*. High-fidelity PCR was used to clone several *PbToxB* loci, which were subsequently confirmed to be homologous to *ToxB*. Additionally, Southern analysis revealed *PbToxB* to have a multicopy nature similar to *ToxB*. A combination of phylogenetic and Southern analyses revealed that the distribution of *ToxB* extends further into the Pleosporaceae, and a search of available fungal genomes identified a distant homolog in *Magnaporthe grisea*, causal agent of rice blast. Thus, unlike other described HSTs, *ToxB*-like sequences are present across a broad range of plant pathogenic ascomycetes.

Introduction

Host-selective toxins (HSTs), a diverse set of molecules known only to be produced by plant pathogenic fungi, function as essential disease determinants in a number of plant host-pathogen interactions (reviewed in Markham and Hille 2001, Walton 1996, Wolpert et al. 2002). They are implicated in pathogenesis/virulence because they are toxic only to hosts susceptible to the fungus, and in turn, toxin production by the pathogen is strictly correlated with disease elicitation on a susceptible host. For a number of HSTs, causality in disease has been molecularly confirmed (Akamatsu et al. 1997, Churchill et al. 2001, Ciuffetti et al. 1997, Johnson et al. 2001, Panaccione et al. 1992, Tanaka et al. 1999, Yang et al. 1996, Yoder et al. 1997). HSTs range from protein products of single genes to low-molecular weight products of multifunctional enzymes or complex enzymatic pathways encoded for by complex gene


Variable distribution of these three HSTs among different isolates of _P. tritici-repentis_, both singly and in all possible combinations, defines a complex race structure for this fungus. Eight races, each with a unique complement of Ptr ToxA, Ptr ToxB, and Ptr ToxC, and a correspondingly unique host range, have been formally described (Lamari et al. 1995, Lamari et al. 2003, Strelkov et al. 2002). Races 1, 2, 7, and 8 produce Ptr ToxA; races 5, 6, 7, and 8 produce Ptr ToxB; and races 1, 3, 6, and 8 produce Ptr ToxC. Nonpathogenic race 4 isolates do not produce any of these three HSTs. Two
new races, race 9 and 10, which produce phytotoxic activities distinct from Ptr ToxA, Ptr ToxB, and Ptr ToxC, were recently described (Chapter 2). Characterization of the previously undescribed activity, from race 9, tentatively named Ptr ToxD (Ciuffetti et al. 2003), is currently in progress in our laboratory (Pandelova and Ciuffetti 2005), and additional races and HSTs are under investigation in other laboratories (Ali and Francl 2002, Ali et al. 2002, Meinhardt et al. 2003).

Although the ToxA gene is present only in Ptr ToxA-producing races of P. tritici-repentis (Lichter et al. 2002), as is the case for other described HSTs, the ToxB gene is unique in that related genes are found in P. tritici-repentis races that do not produce Ptr ToxB (Chapter 2, Martinez et al. 2004, Strelkov et al. 2006), nor are pathogenic on Ptr ToxB-sensitive wheat (Lamari and Bernier 1989a, Lamari et al. 1995). In particular, P. tritici-repentis races 3 and 4 each contain single-copy homologs of ToxB. All but the first six nucleotides are shared between the wild-type ToxB open reading frame (ORF) and its homolog in race 3; however, upstream sequences are distinct (Strelkov et al. 2006). Matching ToxB homologs, which share 86% identity with ToxB, have been identified in two different P. tritici-repentis race 4 isolates, isolate SD20 (Martinez et al. 2004), within which the homolog was named toxb, and isolate 90-2 (Strelkov et al. 2006). Although some of the DNA flanking these homologs is shared with ToxB, the majority of the flanking sequences are unique to these loci (Martinez et al. 2004, Strelkov et al. 2006). The transcriptional regulation of the ToxB homologs in P. tritici-repentis races 3 and 4 is different than that of ToxB (Martinez et al. 2004, Strelkov et al. 2006), presumably due to the presence of distinct upstream sequences. Other non-Ptr ToxB-producing races of P. tritici-repentis do not possess ToxB homologs.

The closest relative to P. tritici-repentis is its sister-species P. bromi (Zhang and Berbee 2001), the causal agent of brown leaf spot of smooth bromegrass (Bromus inermis) (Chamberlain and Allison 1945). Smooth bromegrass (hereafter referred to as bromegrass) is considered one of the best cool-season pasture grasses and is one of two bromegrass species cultivated for permanent pastures to any extent in North America (Vogel et al. 1996). Throughout the bromegrass-growing regions of the U.S. and Canada,
brown leaf spot is one of the most widespread and destructive diseases of this forage crop (Berkenkamp 1974, Elliott 1962, Gross et al. 1975, Kaufmann et al. 1961, Roberts et al. 1955, Smith 1969, Smith and Knowles 1973, Vogel et al. 1996). Susceptibility to brown leaf spot is prevalent among commonly grown cultivars of bromegrass (Zeiders and Sherwood 1986), and like *P. tritici-repentis* in its interaction with wheat, susceptibility to *P. bromi* is dominant to resistance (Berg et al. 1983). The presence of conspicuous chlorotic halos beyond the boundaries of fungal hyphae suggests the involvement of a phytotoxin in disease (Sherwood 1996). Because of the relatedness of *P. bromi* and *P. tritici-repentis* (Zhang and Berbee 2001), and that of their grass hosts (Barker et al. 2001, Hsiao et al. 1994), it is possible that *P. bromi* evolved similar disease mechanisms as *P. tritici-repentis*. Thus, we initiated this study to determine if sequences homologous to the HST genes of *P. tritici-repentis* are present in *P. bromi*. Not only are ToxB homologs present in *P. bromi*, which we designated *PbToxB*, Southern analysis mapped on a phylogeny of *P. tritici-repentis* and its close relatives suggests the distribution of ToxB-like sequences extends throughout the genus *Pyrenophora*, into other genera of the Pleosporaceae. Additionally, a search of available fungal genomes revealed a homolog in a member of another class of the Ascomycota.

**Materials and Methods**

**Fungal isolates**

Isolates used for phylogenetic analyses are listed in Table 3.1. The following *P. bromi* isolates were further analyzed: TW123, TAM115, MPK, SM20A, SM106, SM101, and Bf-1. All *P. bromi* isolates (graciously provided by Drs. Francl and Ali, North Dakota State University) were obtained from smooth bromegrass in Minnesota, except TW123, which was obtained in North Dakota. The *P. tritici-repentis* controls included were: SD8, a race 1 isolate that contains ToxA and produces Ptr ToxA (Chapter 2); DW7, a race 5 isolate that contains ToxB and produces Ptr ToxB (Ali et al. 1999); and SD20, a race 4 isolate that contains the ToxB homolog toxb and does not produce Ptr ToxB.
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^Sequences for two isolates were combined for analysis (see Materials and Methods).

bIsolates for which *MAT*-2 HMG box sequence was missing.

c*P. bromi* 94-1 was revealed to be misidentified, thus was renamed *P. avenae* 94-1b.

d*P. avenae* 98-2 was revealed to be misidentified, thus was renamed *P. teres* 98-2a.

eITS = AF494275; *gpd* = AY004667; *MAT*-2 HMG box = AY004673.

fITS = AF071329; *gpd* = AF081385; *MAT*-2 HMG box = AF275374.

gITS = AF071331; *gpd* = AF081386; *MAT*-2 HMG box = AF032369.

hITS = AF071343; *gpd* = AF081397; *MAT*-2 HMG box = AY335166.

iITS = AF071344; *gpd* = AF081398; *MAT*-2 HMG box = AY335165.

jITS = AF071345; *gpd* = AF081399; *MAT*-2 HMG box = AY335164.

kITS = PNU77362; *gpd* = AY364461; *MAT*-2 HMG box = AY072934.

lCBS = Centraalbureau voor Schimmelcultures.

**Culture conditions**

Fungal cultures were grown on solid V8 juice agar (20% v/v V8 juice, 0.3% w/v CaCO₃, 2% w/v Bacto agar) in constant darkness, 25°C. *P. tritici-repentis* sporulation was induced by flooding a ~4-day-old culture with sterile water and manually depressing aerial hyphae with a glass rod, followed by incubations of 18-24 h of light at room temperature and 18-24 h of darkness at 16°C (Lamari and Bernier 1989a). *P. bromi* sporulation was induced by incubating a ~4 day-old culture in a 12 hr light-12 hr dark cycle for ~5 days (Zeiders and Sherwood 1986). Mycelia for DNA extraction were grown in 75 ml quarter-strength potato dextrose broth (BD Difco, Sparks, MD) for Southern analyses or were scraped from V8-grown cultures for multiplex PCR, followed by lyophilization.

**Plant material and inoculations**

To show representative disease reactions for *P. tritici-repentis*, isolate SD8 was inoculated on the Ptr ToxA-sensitive wheat differential Glenlea (Lamari and Bernier 1989a) and isolate DW7 was inoculated on the Ptr ToxB-sensitive wheat differential 6B662 (Lamari et al. 1995). For *P. bromi*, a representative disease reaction was provided by inoculation of isolate SM101 on the smooth bromegrass cultivar Baylor (U.S. National Plant Germplasm System, USDA, ARS). Prior to inoculation, wheat plants were grown in soil for two to three weeks and smooth bromegrass plants were grown in a peat-vermiculite (1:1) mix for four to five weeks under diurnal conditions of light for 16 h at 23°C and dark for 8 h at 19°C. Inoculations were done with a conidial suspension from each isolate to drip stage, incubated overnight in a dark dew chamber at ~20°C to 22°C, then returned to diurnal conditions. Conidial suspension concentrations were 3x10³
(Lamari and Bernier 1989a) and 1.25×10³ (Zeiders and Sherwood 1986) conidia/ml for P. tritici-repentis and P. bromi, respectively. Disease development was subsequently monitored and secondary or tertiary leaves for P. tritici-repentis and P. bromi, respectively, were harvested 5 to 6 days post-inoculation to document the infection phenotype. Inoculations were repeated multiple times with similar results.

**Multiplex polymerase chain reaction (PCR)**

Methods for genomic DNA extraction via modified instructions for the FastDNA Kit (Qbiogene, Carlsbad, CA) and for multiplex PCR have previously been described (Chapter 2). At least two independent DNA samples for each isolate were analyzed via multiplex PCR.

**Isolation of PbToxB from P. bromi via high-fidelity PCR**

DNA was extracted as for multiplex PCR. Due to its high fidelity, Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) was used to amplify various PbToxB loci from genomic DNA of P. bromi. Primers TB10F and TB12R (Martinez et al. 2004, Martinez et al. 2001) complement sequences outside of the ToxB ORF, thus provided the opportunity to clone the entire ORF with additional flanking sequence. The reaction mixture was composed of 1× High Fidelity PCR Buffer (18 mM ammonium sulfate, 60 mM Tris-SO₄, pH 8.9), 2 mM MgSO₄, 200 μM each dNTP, 200 nM each primer, 1 U Platinum® Taq High Fidelity, and 10 ng template DNA in 25 μl. Amplification was carried out as follows: 1 cycle of 94°C for 1 min; 31 cycles of 94°C for 20 s, 55°C for 20 s, 68°C for 2 min 20 s; 1 cycle of 68°C for 7 min. Amplification products were ligated with pGEM-T Easy (Promega, Madison, WI) and transformed into *Escherichia coli* strain XL-1 Blue. Transformation reactions were screened by PCR and positive clones subjected to sequence analysis (Central Services Laboratory [CSL], Center for Genome Research and Biocomputing [CGRB], Oregon State University [OSU], Corvallis, OR). For each PbToxB locus, four to fourteen clones from two to three independent High Fidelity PCR reactions were sequenced and their sequences compared.
via ClustalW alignment (Thompson et al. 1994). Each PbToxB sequence was confirmed either by exact match or consensus.

**Southern analyses**

Genomic DNA was extracted via the method of Martinez and colleagues (2004) in conjunction with a modified polysaccharide precipitation method (Michaels et al. 1994). DNA concentrations were determined with the Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen, Carlsbad, CA) per a modification of manufacturer’s instructions in conjunction with a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). A 1:150 dilution of the picogreen reagent was used instead of the recommended 1:200 dilution. Quantification of each DNA sample was done in duplicate.

Methods for Southern analyses were as described by Andrie and colleagues (Chapter 2) with certain modifications. The restriction enzymes HindIII and EcoRV were chosen because they do not cut within ToxB or its known homologs. Approximately 1 µg of either HindIII- or EcoRV-digested genomic DNA was size-fractionated followed by downward transfer onto Hybond-N+ membranes (GE Healthcare Life Sciences, Piscataway, NJ) via alkaline transfer. Blots of *P. bromi* were probed with ToxB (GenBank accession number AY007692) or ToxA (GenBank accession number AF004369). Blots of Pleosporalean relatives were probed with either a mix of ToxB, toxb (GenBank accession number AY083456), and PbToxB (represented by PbToxB5-1 from SM106), chosen due to suspected divergence, or ToxA. ToxA probes were amplified as described. ToxB, toxb, and PbToxB probes were amplified with the same reaction conditions as those used for ToxA, except with primers TB16F (Martinez et al. 2001) and TB17R (5'-TATAGAATTCCCTTGTAGAAATGGC-3'), and with the following amplification program: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 35 s, 72°C for 55 s; 1 cycle of 72°C for 7 min.

Multiple hybridization conditions were used for Southern analysis. Blots of *P. bromi* were hybridized at 60°C, overnight, followed by three washes with 2x SSC (0.3 M
NaCl, 0.03 M trisodium citrate), 0.5% SDS at 60°C for 5, 10 and 30 min, and a final high
stringency wash with 0.2× SSC (0.03 M NaCl, 0.003 M trisodium citrate), 2% SDS at
60°C for 45 min. Blots of Pleosporalean relatives were first subjected to low stringency
conditions: prehybridation at 50°C for at least 2 h followed by hybridization with labeled
probe at 50°C overnight. Hybridization was followed by three low-stringency washes
with 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate), 0.5% SDS at 50°C for 5, 10 and 30
min, and a mid-stringency wash of 1× SSC, 0.5% SDS at 50°C and a high stringency
wash of 0.2× SSC, 2% SDS at 65°C, each for 45 min. Blots were exposed to
autoradiographic film at each stringency (Denville Scientific, Metuchen, NJ). At least
two independent DNA samples for each isolate were analyzed via Southern analysis.

Amplification, sequencing, and alignment of ITS, gpd, and MAT-2 HMG box sequences

DNA was extracted as listed above for multiplex PCR. For PCR amplification of
the internal transcribed spacers (ITS), including ITS1, the 5.8S ribosomal RNA gene, and
ITS2, we used the standard primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et
al. 1990). For PCR amplification of a glyceraldehyde-3-phosphate (gpd) fragment
including 450 bp of coding sequence and about 160 bp representing two introns, primers
were as described by Berbee and colleagues (1999). Reaction conditions for both were as
follows: 1× Taq polymerase buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH
9.0), 200 μM each dNTP, 600 nM each primer, 1.25 U Taq polymerase (MBI Fermentas,
Hanover, MD), and 10 ng template DNA in 50 μl. Amplification of ITS was as follows:
1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 45 s; 5
cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s; 1 cycle of 72°C, 5 min.

Amplification of gpd was as follows: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 1
min, 62.7°C for 1 min, 72°C for 45 s; 1 cycle of 72°C, 5 min. All PCR products were
purified on MinElute Spin Columns (Qiagen, Valencia, CA) and directly sequenced.

For amplification of the MAT-2 high mobility group (HMG) box, the degenerate
ChHMG1 and ChHMG2 primers of Arie and colleagues (1996) were 5'-terminally fused
to M13 forward and reverse primers, respectively, which allowed for direct sequencing of
amplification products. The new primers were named M13F-ChHMG1 (5'-TGTAAACCACGAGACCAGTAAGGCNCCNCGYCCNATGAAC-3') and M13R-ChHMG2 (5'-CAGGAAACAGCTATGACCCTNGGNGTGTAYTTGTAATTNGG-3'). The reaction mixture was composed of 1× Taq DNA polymerase buffer B (50 mM KCl, 10 mM Tris·HCl, pH 9.0), 2.5 mM MgCl₂, 200 µM each dNTP, 2 µM each degenerate primer, 2.5 U Taq DNA polymerase (Fisher Scientific, Pittsburgh, PA), and 30 ng template DNA in 50 µl. Amplification was as follows: 1 cycle of 94°C for 3 min; 5 cycles of 94°C for 1 min, 47°C for 30 s, 72°C for 1 min; 25 cycles of 94°C for 1 min, 65°C for 30 s, 72°C for 1 min; 1 cycle of 72°C, 10 min.

In an effort to obtain MAT-2 HMG box sequence from isolates where the aforementioned primers failed to amplify, MAT-2 HMG box primers were redesigned to decrease primer degeneracy. Sequences from successful Pyrenophora MAT-2 HMG box amplifications were used to design primers M13F-Pyren-HMG-For (5'-TGTAAACCACGACCCAGTAAGGCNCCNCGCCTCCGATGAAC-3') and M13R-Pyren-HMG-Rev (5'-CAGGAAACAGCTATGACCCTNGGNGTGTAYTTGTAATTNGG-3') for use with isolates of Pyrenophora. Additionally, the MAT-2 sequence from Alternaria alternata Japanese pear pathotype (GenBank accession number AB009452) was used to design primers M13F-Alt-HMG-For (5'-TGTAAACCACGACCCAGTAAGGCNCCNCGCCTCCGATGAAC-3') and M13R-Alt-HMG-Rev (5'-CAGGAAACAGCTATGACCCTNGGNGTGTAYTTGTAATTNGG-3') for use on distant relatives of Pyrenophora. Despite our efforts, we were still unable to amplify the MAT-2 HMG box for every isolate (see Table 3.1). Additionally, as C. sativus ND93-1 (Zhong and Steffenson 2001b) is mating type 1, it lacks the MAT-2 HMG box (Coppin et al. 1997).

Both DNA strands were sequenced with BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Foster City, CA) on an ABI Prism 3730 or 3730×I Genetic Analyzer by either the CSL of the CGRB at OSU or Macrogen, Inc. (Seoul, Korea), respectively. PCR amplification primers were used as sequencing primers for ITS and gpd; M13F and M13R were used as sequencing primers for the MAT-2 HMG
box. Contigs were assembled in SeqMerge of the GCG software suite (Accelrys, San Diego, CA). Additional sequences were obtained from GenBank (Table 3.1). Sequences were aligned with ClustalW and refined manually in BioEdit 7.0.5 (Hall 1999). Regions of ambiguous alignment were excluded from analyses.

**Data set for phylogenetic analyses**

Our phylogenetic analyses were based on the combined data set of ITS, *gpd*, and the *MAT-2* HMG box for 57 taxa, nine of which were missing *MAT-2* HMG box sequence data (Table 3.1). For the outgroup *Stagonospora nodorum*, data was combined from two different isolates. Sequences taken from GenBank for ITS (PNU77362) and *gpd* (AY364461) originating from isolate S74-20A were combined with the *MAT-2* HMG box (AY072934) sequence from isolate Sn26-1. In order to determine if these *S. nodorum* sequences could be combined and considered as one taxon for our analyses, ITS and *gpd* sequences from S74-20A and Sn26-1 were compared to each other. The ITS sequences for *S. nodorum* isolates S74-20A and Sn26-1 were identical and their *gpd* sequences had one base pair difference confirming their conspecificity (Dr. Peter P. Ueng, USDA-ARS, BARC-West, personal comm.). Additionally, available ITS (AF071329) and *gpd* (AF081385) sequences for *C. sativus* from isolate A20 (Berbee et al. 1999) were combined with a *MAT-2* HMG box (AF275374) sequence from isolate ND90Pr (Zhong and Steffenson 2001b). Sequences were not available such that a cross-sequence comparison could be made for *C. sativus* like that done for *S. nodorum*, however we included this complete set of sequences for *C. sativus* because our *C. sativus* isolate is of the opposite mating type. Subsequent assessment of sequence data congruence provided support for our decision.

**Phylogenetic analyses**

Phylogenetic analyses included maximum and weighted parsimony (MP and WP, respectively) as implemented by PAUP* version 4b10 (Swofford 2002) and Bayesian inference of maximum likelihood as implemented by MRBAYES (Huelsenbeck and
Ronquist 2001). For MP and WP analyses, the final data set included 1752 characters (709 for ITS, 797 for gpd, and 246 for the MAT-2 HMG box), 636 of which were excluded due to ambiguous alignment (234 for ITS, 347 for gpd, and 55 for the MAT-2 HMG box). Of the remaining 1116 characters (475 for ITS, 450 for gpd, and 191 for the MAT-2 HMG box), 206 were parsimony informative (53 for ITS, 70 for gpd, and 83 for the MAT-2 HMG box). Heuristic searches were run according to the following parameters: TBR branch swapping and MulTrees were on, starting trees were obtained by stepwise addition of 100 random replicates, MaxTrees was set at 10,000, and gaps were treated as missing. For WP analyses, the data set was partitioned according to locus, and symmetric step matrices generated with STMatrix 2.2.1 (F. Lutzoni and S. Zoller, Duke University; software available at http://www.lutzonilab.net) were based on the unambiguously aligned sequences of those taxa not missing MAT-2 HMG box sequence data. However, the data was not partitioned according to codon due to the low number of characters and inclusion of non coding characters from MAT-2 HMG box sequence. Relative support for the resulting trees was determined with 100 bootstrap replicates (Felsenstein 1985) performed with the aforementioned heuristic search options.

Given the limited set of models available in MRBAYES and because it has been shown that parameter-rich models generally perform better than other models in Bayesian analysis, we chose a general time-reversible model with a proportion of invariant sites and a gamma distribution for among site variation (GTR + \gamma + I) for Bayesian analysis. As for WP analysis, the data was partitioned according to locus and not by codon. A Markov Chain Monte Carlo (MCMC) analysis was run starting from a random tree for \(4 \times 10^6\) generations, sampling every 100th cycle. Out of the 40,000 resulting trees, the initial 4000 trees were identified as burn in prior to the convergence of likelihoods and thus were excluded from post-run analyses. Three independent analyses were conducted simultaneously to verify likelihood convergence and burn in parameter. The harmonic mean clustered around a likelihood value of -11987. A 50% majority rule consensus tree of 36,000 Bayesian likelihood trees was subsequently constructed, and average branch lengths and posterior probabilities determined.
Evaluation of sequence data congruence

Combinability of ITS, gpd, and MAT-2 HMG box sequence data was first evaluated by manual comparisons of 70% MP trees for each gene as implemented by PAUP* version 4b10 as described above. This was completed both for a 48 taxa data set that included those taxa with sequence available for all three loci and for an expanded 57 taxa data set that included an additional nine taxa that lacked MAT-2 HMG box sequence data to make sure their addition did not significantly alter tree topology. Concordance of the combined data was also evaluated with 100 partition homogeneity test (PHT)-replicates as implemented by PAUP* version 4b10 with settings as described above for WP. For the 48 taxa data set, all three loci were included, whereas for the expanded 57 taxa data set, only sequence data for ITS and gpd was included due to missing MAT-2 HMG box sequence data.

Results

P. bromi contains ToxB-like sequences, designated PbToxB

In light of the relatedness of P. tritici-repentis and P. bromi (Zhang and Berbee 2001), and that of their grass hosts (Baker et al. 2006, Hsiao et al. 1994), comparison of disease phenotypes resulting from inoculation of P. tritici-repentis on sensitive wheat (Figure 3.1A) with that of inoculation of P. bromi on bromegrass (Figure 3.1B) raised the question as to whether P. bromi might produce toxins homologous to Ptr ToxA or Ptr ToxB from P. tritici-repentis. A multiplex PCR screen with primers to both ToxA and ToxB (Chapter 2) was used to screen seven isolates of P. bromi as compared to P. tritici-repentis (Figure 3.1C). P. tritici-repentis controls, including ToxA-positive race 1 isolate SD8 (Figure 3.1C, lane 8) and ToxB-positive race 5 isolate DW7 (Figure 3.1C, lane 9), behaved as expected and produced ToxA and ToxB amplification products, respectively. A ToxB-like product, but not a ToxA-like product, was amplified from all seven isolates of P. bromi (Figure 3.1C). Amplification of a CHS-1 product served as an internal control for the presence of fungal DNA. According to P. tritici-repentis HST nomenclature (Ciuffetti et al. 1998), we designated these ToxB-like loci as PbToxB, and
Figure 3.1. Disease symptoms caused by *Pyrenophora tritici-repentis* and *P. bromi* on wheat and bromegrass, respectively, and the discovery of *ToxB*, a host-selective toxin gene from *P. tritici-repentis*, in *P. bromi*.

A) Inoculation of Ptr ToxA-producing and Ptr ToxB-producing isolates of *P. tritici-repentis* on corresponding susceptible wheat differentials.

B) Inoculation of *P. bromi* on bromegrass. C) Multiplex polymerase chain reaction reveals the presence of *ToxB*-like, but not *ToxA*-like, sequences in seven isolates of *P. bromi* (lanes 1-7). *P. tritici-repentis* isolates 86-124 (lane 8) and DW7 (lane 9) provide positive controls for the presence of *ToxA* and *ToxB*, respectively. Chitin synthase 1, CHS-1, serves as an internal control for the presence of fungal DNA. *Ptr* = *P. tritici-repentis*, *Pb* = *P. bromi*, 1 = TW123, 2 = TAM115, 3 = MPK, 4 = SM20A, 5 = SM106, 6 = SM101, 7 = Bf-1.
the corresponding proteins as Pb ToxB.

Southern analysis of PbToxB in P. bromi

The presence of PbToxB in the genome of P. bromi was confirmed via Southern analysis (Figure 3.2A). When HindIII-cut genomic DNA was probed with the ToxB open reading frame (ORF), hybridization patterns were as expected for P. tritici-repentis (Martinez et al. 2004). ToxB was found in multiple copies in pathogenic race 5 isolate DW7 (Figure 3.2A, lane 8) and toxb was found as a single copy in nonpathogenic race 4 isolate SD20 (Figure 3.2A, lane 9). ToxA-positive race 1 isolate SD8 provided a negative control (Figure 3.2A, lane 10). For the various isolates of P. bromi, from one to three PbToxB bands were observed (Figure 3.2A, Lanes 1-7). P. bromi isolates TW123 (Figure 3.2A, lane 1) and TAM115 (Figure 3.2A, lane 2) had one band each, Bf-1 had three bands (Figure 3.2A, lane 7), and the rest had two bands (Figure 3.2A, lanes 3-6). Thus, like ToxB in P. tritici-repentis, PbToxB in P. bromi was found both as a single copy and in multiple copies, although not to the extent found in race 5 of P. tritici-repentis. When probed with ToxA, only ToxA-positive P. tritici-repentis isolate SD8 produced a hybridization signal (data not shown). In accordance with results of PCR (Figure 3.1), the lack of ToxA signal for P. bromi via Southern analysis confirmed the absence of ToxA in its genome (data not shown). Comparable results were obtained for hybridization of either probe (ToxB or ToxA) to EcoRV-digested genomic DNA (data not shown).

Cloning of various PbToxB loci

High-fidelity PCR analysis with primers outside of the ToxB ORF corroborated the multicopy nature of PbToxB in P. bromi, as well as provided a means to clone the loci for characterization (Figure 3.2B). Primers TB10F and TB12R (Martinez et al. 2001) amplified a range of fragment sizes from isolates of P. bromi (Figure 3.2B, lanes 1-7). In agreement with Southern analyses (Figure 3.2A, lanes 1-2), only one product each, a
Figure 3.2. ToxB-like sequences are found both as a single copy and as multiple copies in the genome of Pyrenophora bromi.

A) Southern blot analysis of P. bromi (lanes 1-7) as compared to P. tritici-repentis (lanes 8-10). One microgram of HindIII-digested genomic DNA from each isolate was hybridized with a ToxB probe and washed at high stringency. B) Polymerase chain reaction analysis with primers outside of the ToxB open reading frame of P. bromi (lanes 1-7) as compared to P. tritici-repentis (lanes 8-10). P. tritici-repentis isolates DW7 (lane 8) and SD20 (lane 9) serve as positive controls for the presence of ToxB and toxb, respectively. The ToxA-containing P. tritici-repentis isolate SD8 (lane 10) is included as a negative control. Ptr = P. tritici-repentis, Pb = P. bromi, 1 = TW123, 2 = TAM115, 3 = MPK, 4 = SM20A, 5 = SM106, 6 = SM101, 7 = Bf-1. Sizes of bands in the DNA mass ladder are noted to the right.
798 base pair (bp) or a 699 bp fragment, was amplified from the *P. bromi* isolates TW123 (Figure 3.2B, lane 1) and TAM115 (Figure 3.2B, lane 2), respectively. Additionally, the *P. bromi* isolates MPK, SM20A, SM106, and SM101 produced two amplification products each, 699 bp and 749 bp fragments (Figure 3.2B, lanes 3-6). In contrast, although Southern analysis suggested three, only two products were amplified from *P. bromi* isolate Bf-1, 699 bp and 724 bp fragments (Figure 3.2B, lane 7). *ToxB*-positive *P. tritici-repentis* isolate DW7 also only amplified one 689 bp fragment with these primers (Figure 3.2B, lane 8), despite the presence of nine to eleven *ToxB* copies in its genome (Figure 3.2A, lane 8); *toxb*-positive *P. tritici-repentis* isolate SD20 produced one band of 1120 bp in length (Figure 3.2B, lane 9); and *ToxA*-positive isolate SD8 provided a negative control (Figure 3.2B, lane 10). The amplification products from *P. bromi* were cloned and sequenced.

**PbToxB nomenclature**

To manage the variability of *PbToxB* suggested by Southern and PCR analyses, we devised a system to standardize nomenclature for this locus (Yoder et al. 1986). First, each *PbToxB* locus was given a number, starting with 1, to designate its resident isolate. For example, all *PbToxB* loci found in *P. bromi* isolate SM101 were designated *PbToxB6*. When presented with multiple copies of *PbToxB* in one isolate, we then assigned secondary numbers to distinguish between the sequence variants from that isolate. For example, to distinguish between the two loci identified in *P. bromi* isolate SM101, they were designated *PbToxB6-1* and *PbToxB6-2*. If another *PbToxB* locus is subsequently identified in SM101, it will be designated *PbToxB6-3*. These designations will make it obvious whether various *PbToxB* loci share a resident isolate. Table 3.2 provides a summary of the *PbToxB* loci thus far identified. A subset of *PbToxB* loci were subjected to further analyses, including: *PbToxB1* from TW123, *PbToxB2* from TAM115, *PbToxB5-1* and *PbToxB5-2* from SM106, *PbToxB6-1* and *PbToxB6-2* from SM101, and *PbToxB7-2* and *PbToxB7-2* from Bf-1.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Protein</th>
<th>Isolate</th>
<th>TB10-TB12&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lane #&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
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<td>2</td>
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<tr>
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<td><em>Pb ToxB3-1</em></td>
<td>MPK</td>
<td>749</td>
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<tr>
<td><em>PbToxB3-2</em></td>
<td><em>Pb ToxB3-2</em></td>
<td>MPK</td>
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<td>3</td>
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<tr>
<td><em>PbToxB4-1</em></td>
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<td>4</td>
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<tr>
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<tr>
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<td><em>Pb ToxB7-1</em></td>
<td>Bf-1</td>
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<td><em>Pb ToxB7-2</em></td>
<td>Bf-1</td>
<td>699</td>
<td>7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sizes of TB10-TB12 PCR fragments are given in base pairs.

<sup>b</sup>Lane # = For convenience, locus numbers correspond to lane numbers in Figures 3.1 and 3.2.

<sup>c</sup>The *Pb ToxB1* and *Pb ToxB5-1* deduced amino acid sequences are identical, although their corresponding ORF sequences differ by one nucleotide.

<sup>d</sup>The *PbToxB2*, *PbToxB7-1*, and *PbToxB7-2* ORFs, and the proteins they encode, are identical to each other.
Sequence comparisons between loci from *P. bromi* and *P. tritici-repentis*

The *PbToxB* ORFs are 264 bp in length like *toxb* (Martinez et al. 2004), corresponding to the insertion of one codon as compared to the 261 bp *ToxB* ORF (Martinez et al. 2001) (data not shown). Concurrently, the amino acid (aa) sequence of *Pb ToxB*, like *Ptr toxb* from *P. tritici-repentis* race 4 isolate SD20, is 88 aa in length (Martinez et al. 2004), corresponding to the insertion of one amino acid as compared to the 87 aa *Ptr ToxB* protein from *P. tritici-repentis* race 5 isolate DW7 (Martinez et al. 2001, Strelkov et al. 1999) (Figure 3.3), which is predicted by ClustalW multiple sequence alignment (Thompson et al. 1994) to be at position 72 (Figure 3.3A, ^). Interestingly, *Ptr toxb* possesses a proline at this position, an amino acid which is known to alter protein structure, whereas the *Pb ToxB* proteins do not. Additionally, all three *ToxB* homologs (*PbToxB, ToxB, and toxb*) encode a conserved signal peptide (Figure 3.3A, black line) predicted by SignalP 3.0 (Bendtsen et al. 2004) to be cleaved after the alanine (Figure 3.3A, boxed A) at position 23 (Figure 3.3A, ▼), and share four conserved cysteines predicted by Disulfind (Vullo and Frasconi 2004) to be involved in disulfide pairing (Figure 3.3A, *).

Pairwise sequence comparisons of *ToxB, toxb, and PbToxB*, and of the corresponding amino acid sequences of *Ptr ToxB,Ptr toxb, and Pb ToxB*, with the local BLAST (Altschul et al. 1997) function of BioEdit (Hall 1999) unveiled a triangle of identity between these three loci (Figure 3.3B). To account for slight variability between the loci of *P. bromi*, results are given as an average of the individual comparisons for those loci included. *PbToxB* is 87.5% and 87% identical to *ToxB* and *toxb*, respectively, and *ToxB* and *toxb* are 87% identical to each other. Comparisons of deduced amino acid sequences of these loci, with BLOSUM 62 (Henikoff and Henikoff 1992) as the chosen scoring matrix, revealed that the full-length *Pb ToxB* protein is 81.7% and 80.5% identical to the full-length *Ptr ToxB* and *Ptr toxb* proteins, respectively. Additionally, the full-length *Ptr ToxB* and *Ptr toxb* proteins are 81.8% identical to each other. A comparison of the mature protein sequences excluding the signal sequence revealed that *Pb ToxB* is 76.7% and 75.2% identical to *Ptr ToxB* and *Ptr toxb*, respectively, and *Ptr
Figure 3.3. *Pyrenophora bromi* possesses a set of *ToxB* homologs, designated *PbToxB*.

A) ClustalW alignment (Thompson et al. 1994) of deduced *Pb ToxB* amino acid (aa) sequences in comparison to *Ptr ToxB* and *Ptr toxb*. Residue designations are as follows: black on gray = globally conserved; white on black = identical for >50% of sequences; black on white = unique or shared by <50% of sequences; and, white on gray = conservative substitutions. Black line = putative signal sequence. \( \wedge \) = conserved signal sequence cleavage site following alanine (boxed A). * = conserved cysteines. ^ = single aa insertion missing from *Ptr ToxB*. Groups of *Pb ToxB* sequences that are 100% identical to each other are denoted by either # or +. B) Pairwise sequence comparisons of *Ptr ToxB/IPbToxB*, *Ptr toxb/toxb*, and *Pb ToxB/PbToxB*. Results for *Pb ToxB/PbToxB* are given as an average of the results for all eight included *Pb ToxB/IPbToxB* loci. aa = amino acid, nt = nucleotide. The sequences for *Ptr ToxB* (GenBank accession number AY007692) and *Ptr toxb* (GenBank accession number AY083456) were published previously (Martinez et al. 2001, 2004).
ToxB and Ptr toxb are 78.5% identical to each other. Comparison of the corresponding and ToxB and toxb are 87% identical to each other. Comparisons of deduced amino acid sequences of these loci, with BLOSUM 62 (Henikoff and Henikoff 1992) as the chosen scoring matrix, revealed that the full-length Pb ToxB protein is 81.7% and 80.5% identical to the full-length Ptr ToxB and Ptr toxb proteins, respectively. Additionally, the full-length Ptr ToxB and Ptr toxb proteins are 81.8% identical to each other. A comparison of the mature protein sequences excluding the signal sequence revealed that Pb ToxB is 76.7% and 75.2% identical to Ptr ToxB and Ptr toxb, respectively, and Ptr ToxB and Ptr toxb are 78.5% identical to each other. Comparison of the corresponding nucleotide sequence excluding the portion encoding the signal sequence revealed that this region of PbToxB is 85.9% and 84.7% identical to the same stretch of ToxB and toxb, respectively. Likewise, this region of ToxB and toxb is 85.5% identical.

Sequence comparisons within *P. bromi*

Pairwise sequence comparisons between the *P. bromi* loci with the local BLAST function of BioEdit revealed that they are more similar to each other than to either locus, ToxB or toxb, from *P. tritici-repentis* (Figure 3.3). The full-length PbToxB nucleotide sequences are ≥7.8% identical and the corresponding full-length Pb ToxB amino acid sequences including the signal sequence are ≥6.6% identical, which amounts to one or two amino acids differences between the various Pb ToxB proteins. With the signal sequence excluded, the PbToxB nucleotide sequences are ≥7.0% identical and the corresponding PbToxB amino acid sequences are ≥5.4% identical. Although most of the PbToxB genes, and the proteins they encode, possess some level of sequence variation, examples of 100% identity exist both within and between isolates (Figure 3.3A). Both PbToxB loci from *P. bromi* isolate Bf-1, PbToxB7-1 and PbToxB7-2, and the corresponding Pb ToxBs they encode, Pb ToxB7-1 and Pb ToxB7-2, share 100% identity to each other (Figure 3.3, +). These loci also share 100% identity with PbToxB2, and its corresponding protein Pb ToxB2, from *P. bromi* isolate TAM115 (Figure 3.3, +). Additionally, the protein sequence of Pb ToxB5-1 from SM106 matches Pb ToxB1 from
Comparison of sequences flanking *PbToxB*, *ToxB*, and *toxb*

PCR analysis with primers TB10F and TB12R amplified additional sequences upstream (from a minimum of 285 bp for *ToxB* to a maximum of 755 bp for *toxb*) and downstream (98 bp for all loci amplified) of the *ToxB*, *PbToxB*, and *toxb* ORFs. Comparison of the entire amplicon for all loci revealed that, whereas *ToxB* lacks an upstream insertion-deletion (indel) sequence, the presence of variably-sized indel sequences (50 bp, 75 bp, 100 bp, or 150 bp) upstream of the different *PbToxBs* accounts for the size differences observed for the Tb10F-Tb12R PCR amplification products from different isolates of *P. bromi* (Figure 3.2B, Table 3.2). Amplicons of the same size share nearly identical (one nucleotide difference) to identical indel sequences. Additionally, these upstream indel sequences distinguish between the *PbToxB* loci mentioned above that share 100% identity. However, the 699 bp amplification products from TAM115, which contains *PbToxB2*, and from Bf-1, which contains *PbToxB7-2*, are the exception, in that they are identical along their whole length. Although *toxb* also has an upstream indel sequence in a similar position as those upstream of the *PbToxB* loci, the sequence of its indel is distinct (data not shown). Besides indel sequence differences, sequences both upstream and downstream of the *PbToxB*, *ToxB*, and *toxb* ORFs obtained with the Tb10F and Tb12R primers were quite similar, although a few scattered point mutations were present. Expression of these loci may reflect differences in upstream sequences.

Phylogenetic analysis of *P. tritici-repentis* and its Pleosporalean relatives

We conducted a phylogenetic analyses based on a combined data set of ITS, *gpd*, and *MAT-2* HMG box sequences in an effort to establish a hierarchy of *P. tritici-repentis* and its Pleosporalean relatives within which to frame investigation of various evolutionary hypotheses (Figure 3.4A). Additionally, due to the potential that *P. tritici-repentis* and *P. bromi* can be confused based on morphology alone (Krupinsky 1986),
Figure 3.4. *ToxB*-like sequences are present in other members of the Pleosporaceae.

A) One of 932 trees from a weighted parsimony analysis of *Pyrenophora tritici-repentis* and its Pleosporalean relatives based on combined ITS, *gpd*, and *MAT-2* HMG box data. Bootstrap values of 100 replicates for weighted parsimony analysis are shown above branches, followed by the same for maximum parsimony analysis. Posterior probabilities for Bayesian analysis are shown below branches. * = denotes isolates chosen for Southern analysis. # = denotes isolates that were previously shown to possess *ToxB* or *ToxB* homologs (Martinez et al 2004, Strelkov et al 2006). B-D) Southern blot analyses of chosen representatives from each clade. One microgram of either *HindIII*- or *EcoRV*-digested genomic DNA from each isolate was hybridized with a mixture of *ToxB*, *toxb*, and *PbToxB* and washed at mid-stringency. Aa = *Alternaria alternata*, Cs = *Cochliobolus sativus* ND93-1, Cv = *C. victoriae* HVW, Pa 94-1b = *P. avenae* 94-1b, Pa 95-1 = *P. avenae* 95-1, Pl = *P. lolii* 95-1A, Pt = *P. teres* 99-4, Ptm = *P. teres* fsp. *maculata* 1049 WRS, Pg = *P. graminea* 5.
Figure 3.4. *ToxB*-like sequences are present in other members of the Pleosporaceae.
phylogenetic analyses confirmed that our *P. bromi* isolates belong to *P. bromi* and are not misidentified isolates of *P. tritici-repentis* (Figure 3.4A). Support for combination of sequence data for the three loci chosen for analysis was provided by both a lack of conflict between single gene 70% maximum parsimony (MP) analyses and by results of a partition homogeneity test (PHT). Incongruency between loci was rejected (p-value = 0.93) by PHT for all data sets. Addition of taxa with missing *MAT-2* HMG box sequence data did not significantly alter tree topology (data not shown), nor did combining sequences from different isolates of *Cochliobolus sativus* or *Stagnospora nodorum*. *S. nodorum* was the chosen outgroup due to the availability of sequence data from GenBank for all three loci. One of 932 equally parsimonious trees from a weighted parsimony (WP) analysis of the final 57 taxa data set is shown in Figure 3.4A. WP bootstrap values are present above branches, followed by MP bootstrap values. Bayesian analyses produced 46,000 trees (data not shown), the probability numbers from which are given below branches. No significant conflicts were observed between the different methods of analyses. Although higher-order relationships are not well resolved, final trees for all analyses provided significant support for terminal clades.

**Southern analysis reveals the presence of ToxB-like sequences in other relatives of *P. tritici-repentis***

Superimposition of the results of Southern analyses on a phylogram of *P. tritici-repentis* and its Pleosporalean relatives suggested that the distribution of ToxB-like sequences extends throughout the genus *Pyrenophora* and to other genera of the Pleosporaceae (Figure 3.4). As was the case for *PbToxB*, the ToxB-like sequences in these other relatives were shown to be present in multiple copies, including in *Cochliobolus* and *Alternaria* (Figure 3.4B), and other members of the genus *Pyrenophora* (Figure 3.4C and D). Due to the anticipated divergence of ToxB homologs in more distant relatives, less stringent conditions were used than for Southern analysis of *P. bromi* (Figure 3.2B as compared to Figure 3.4B-D). When wash stringencies were increased for Southern blots of more distant relatives, at least one ToxB-positive band remained for all fungi tested, although more prominently for members of *Pyrenophora*.
than for members of other genera in the Pleosporaceae (data not shown). Additionally, although TB10F and TB12R failed to amplify a target from any species other than *P. bromi*, amplification with primers specific to the ToxB ORF was successful in a subset of tested species (data not shown). Sequence analysis of the corresponding amplification products confirmed the presence of additional ToxB sequence variants, although further investigation is necessary (data not shown). Thus, in contrast to other HSTs, our results suggest that the distribution of ToxB not only uniquely extends to different races of the same species, it continues across the members of the *Pyrenophora* to other genera in the Pleosporaceae. However, definitive proof awaits cloning of these putative Toxb-like sequences. Upon subsequent characterization, we suggest designation of these loci follow the convention as was done for PbToxB in *P. bromi*.

A ToxB homolog identified in the Magnaporthe grisea genome

The recent availability of a number of annotated fungal genome sequences (Galagan et al. 2005) allowed us to further investigate the distribution of ToxB. The results of a default BLASTP similarity search (Altschul et al. 1997) of the annotated fungal genomes of the Broad Institute Fungal Genome Initiative (www.broad.mit.edu/annotation/fungi/magnaporthe) with Ptr ToxB as a query and BLOSUM62 as the chosen scoring matrix revealed significant identity to MGG 09686.5, a predicted protein from *Magnaporthe grisea*, causal agent of rice blast (Rossman et al. 1990a) and a member of a different class of the Ascomycota than that to which *P. tritici-repentis* belongs (Eriksson 2005). Prior to further analyses, the automated annotation of this predicted protein was manually assessed.

Of the 79 aa that aligned between the 87 aa of Ptr ToxB and the 88 aa of MGG 09686.5, 31% were identical and 49% were conserved, as determined by both BLASTP and SSEARCH (Pearson 1991, Smith and Waterman 1981), with BLOSUM62 engaged. The homology threshold, or the percentage at which structural homology can be assumed from sequence alignment, is at least 24.8% for a fragment ≥80 aa in length (Sander and Schneider 1991), thus 31% identity for 79 alignable aa provides support for homology
between these two sequences. Comparison of MGG 09686.5 to 1000 shuffled sequences of Ptr ToxB with PRSS (Pearson 1996), resulted in a Smith-Waterman (S-W) score of 104, p-value = 0.001, which is within the values of statistical significance expected for clearly- to distantly-related sequences, which are p-value = 0.0001 and p-value = 0.007, respectively. The converse comparison of Ptr ToxB to 1000 shuffled sequences of MGG 09686.5 gave the same S-W score of 104, although was even more significant, p-value = 0.0006, than the inverse comparison of MGG 09686.5 toPtr ToxB. Additionally, the two amino acid insertions introduced upon alignment of these two sequences (Figure 3.5) fell within regions of secondary structure, as predicted by PHD (Rost 1996) on PredictProtein (Rost et al. 2004) and by PSIPRED (Jones 1999) on the PSIPRED server (Bryson et al. 2005, McGuffin et al. 2000), where they are not expected to be structurally disruptive.

Ptr ToxB and MGG 09686.5 share additional features. SignalP 3.0 predicts the presence of a signal peptide in MGG 09686.5, with a cleavage site (Figure 3.5, ▼) following alanine (Figure 3.5, boxed A) very near that of Ptr ToxB. All four cysteines predicted by Disulfind to form disulfide pairs in Ptr ToxB are conserved in MGG 09686.5 (Figure 3.5, *). Ptr toxb (32.9% over 82 aa) and Pb ToxB6-1 (31.2% over 80 aa) show comparable identities to MGG 09686.5 as that of Ptr ToxB (31.6% over 79 aa), as would be expected considering the triangle of identity between Ptr ToxB, Ptr toxb, and Pb ToxB (Figure 3.3B). Upon further characterization, we suggest that the ToxB homolog in M. grisea be designated as MgToxB, with a corresponding protein name of Mg ToxB. At this time, no other significant matches were found in any of the other available fungal genomes (www.broad.mit.edu/annotation/fungi; www.ncbi.nlm.nih.gov), even with less strict search parameters.

Discussion

The genetic determinants for HSTs are typically present only in pathogenic isolates, without a corresponding allele in nonpathogenic isolates. ToxB was formerly shown to be unique as a HST because ToxB homologs are found in races of P. tritici-repentis that do not produce the corresponding protein, Ptr ToxB (Martinez et al. 2004).
Figure 3.5. A homolog of Ptr ToxB, predicted protein MGG 09686.5, is present in the Magnaporthe grisea genome.

CGRB Align 2, with SSEARCH (Smith and Waterman 1981, Pearson 1991) and BLOSUM62 (Henikoff and Henikoff 1992) engaged, was used to align Ptr ToxB and MGG 09686.5. Identical amino acids are highlighted in black and conserved amino acids are highlighted in gray. Black lines = putative signal sequences. ∗ = conserved signal sequence cleavage sites following alanine (boxed A). * = conserved cysteines. Unalignable residues of each open reading frame are presented in lowercase characters. P = Pyrenophora tritici-repentis, M = M. grisea.
The results of this study suggest that the distribution of ToxB extends even further than originally anticipated. Not only are ToxB homologs found in sister-species *P. bromi* (Figures 3.1-3.3), the results of Southern analysis indicate they are likely found throughout the *Pyrenophora* and in other genera of the Pleosporaceae, including *Cochliobolus* and *Alternaria* (Figure 3.4). A ToxB homolog was also identified in an even more distantly-related fungus, *M. grisea* (Figure 3.5). Thus, unlike other described HSTs, ToxB is present across a broad range of plant pathogenic ascomycetes. The homologous genes *ChPKSJ* and *MzmPKSJ*, the products of which are involved in the production of T-toxin by *C. heterostrophus* and PM-toxin by *Didymella zeae-maydis* (formerly *Mycosphaerella zeae-maydis* and also known as *Pyllosticta maydis*), respectively, are the only other HST genetic determinants known to be distributed to more than one species (Baker et al. 2006, Kroken et al. 2003, Yoder 1998, Yun et al. 1998).

Despite that some races of *P. tritici-repentis* themselves lack ToxB, ToxB-like sequences were found in all tested *P. bromi* isolates and designated *PbToxB* (Figures 3.1 and 3.2). The high sequence identity between ToxB, toxb, and *PbToxB*, in addition to the presence of other shared features, reveals obvious homology between these sequences (Figure 3.3). The flanking sequences obtained with the TB10F and TB12R primers for these loci were also very similar, with the exception of the presence of indel sequences upstream of *PbToxB* and toxb that are absent upstream of ToxB. The differences in flanking sequences between ToxB and the various *PbToxB* loci are reflected in a comparison of the results of Southern analysis (Figure 3.2A) with those of PCR analysis with the TB10F and TB12R primers (Figure 3.2B). Due to identical sequences upstream of all ToxB loci in *P. tritici-repentis* isolate DW7 up to where TB10F anneals (Martinez et al. 2004), only one PCR product amplifies with these primers despite the presence of 9-11 copies of ToxB as shown by Southern analysis. In contrast, PCR analysis reflects Southern analysis with regard to copy number for the isolates of *P. bromi* due to the presence of these variably-sized indel sequences upstream of the different *PbToxB* ORFs in this same region. *P. bromi* isolate Bf-1 is the one exception, in that Southern analysis
indicates 3 bands whereas PCR only indicates two, which suggests that at least two of its PbToxB loci share similar flanking sequences.

Based on observations that race 4 isolates make up 98% of the P. tritici-repentis races found on noncereal grasses, but only 5% of those found on wheat (Ali and Francl 2003b), Martinez and colleagues (2004) suggested that toxb might be involved in the interaction of P. tritici-repentis race 4 isolates on other grass hosts. It is also quite possible that PbToxB is involved in the interaction of P. bromi with bromegrass. Not only are P. tritici-repentis and P. bromi sister-species (Figure 3.4) (Zhang and Berbee 2001), so are their respective hosts Triticum and Bromus (Barker et al. 2001, Hsiao et al. 1994). As such, it is possible that these two organisms experienced similar selection pressures and thus PbToxB may encode for an HST in this sister disease interaction. The disease phenotype of P. bromi on bromegrass corroborates this premise (Figure 3.1B), as it is suggestive of the involvement of an HST in disease (Sherwood 1996) and is similar to that of P. tritici-repentis on wheat (Figure 3.1A). That multiple copies of PbToxB have been maintained in the P. bromi genome (Figure 3.2) provides additional support that PbToxB may function similarly to ToxB. Inoculation of nonpathogenic P. tritici-repentis race 4 transformants containing variable numbers of ToxB under the control of the same constitutive promoter revealed a positive correlation between ToxB copy number and chlorotic symptom development, which suggests that multiple copies of ToxB, or at least high levels of Ptr ToxB production, are necessary to cause disease on Ptr ToxB-sensitive wheat (Martinez and Ciuffetti, unpublished data). The possibility that PbToxB acts as an HST in the interaction between P. bromi and bromegrass is under further investigation.

Although a Pyrenophora phylogeny based on ITS and gpd was published by Zhang and Berbee (Zhang and Berbee 2001) during the course of this study, we completed our own phylogenetic analysis such that biological questions subsequently could be asked of isolates anchored in the hierarchy. We included an additional locus, the MAT-2 HMG box, due to its proposed superior resolving power among closely-related lineages (Turgeon 1998, Turgeon and Berbee 1998) and to better represent the
species phylogeny with use of multiple loci. Although our analysis encompassed a narrower overall sampling than previous efforts, our results were congruent with those available for Pyrenophora (Zhang and Berbee 2001), Cochliobolus (Berbee et al. 1999), Pleospora (Câmara et al. 2002), and Alternaria (Pryor and Bigelow 2003), all of which form monophyletic clades. As suggested, inclusion of the MAT-2 HMG box provided more phylogenetic signal, as measured by parsimony informative characters per base pair (83 bp out of 264 bp), than either ITS (53 bp out of 709 bp) or gpd (70 bp for 798 bp), however this increase in phylogenetic signal did not translate into a substantial increase in resolution to that obtained with ITS and gpd alone (compare Figure 3.4 with Zhang and Berbee [2001]). Although phylogenetic analyses of Colletotrichum (Du et al. 2005), Ascochyta (Barve et al. 2003), Ceratocystis (Withthuhn et al. 2000), and Calonectria (Schoch et al. 2000) found the MAT-2 HMG box more useful than ITS for discriminating among closely related lineages, the results of O’Donnell and colleagues (2004) suggest that MAT genes are no more phylogenetically informative than most other genic regions and thus urge further investigation to ascertain what region(s) of MAT might be the most phylogenetically informative. Indeed, phylogenetic analyses of the Gibberella fujikuroi (Steenkamp et al. 2000) and Leptosphaeria maculans (Voigt et al. 2005) species complexes suggest the 3'-idiomorph flank of MAT-2 is better suited for resolving close relationships, possibly even intraspecific differences (Voigt et al. 2005).

Results of Southern analyses charted on a phylogram of P. tritici-repentis and its Pleosporalean relatives suggest additional ToxB homologs are present throughout the genus Pyrenophora, as well as in members of Cochliobolus and Alternaria (Figure 3.4). Although definitive confirmation awaits subsequent identification and characterization of the putative ToxB-like sequences from these other species, preliminary sequence analysis of PCR products amplified with ToxB ORF-specific primers confirmed the presence of ToxB sequence variants in a few tested species (data not shown). A ToxB homolog, MGG 09686.5, was also identified in the genome of M. grisea, further extending the boundary of the known distribution of ToxB (Figure 3.5). Thus, in contrast to other HSTs, for which horizontal gene transfer has been evoked to explain their irregular
distribution, the broad distribution of _ToxB_ appears to be like that of a gene vertically transferred from a common ancestor as opposed to one horizontally acquired. As was proposed for the distribution of type I polyketide synthase genes in ascomycetes (Kroken et al. 2003), including the HST determinants _ChPKSI_ and _MzmPKSI_, the current distribution of _ToxB_ can be readily explained by gene duplication, divergence, and loss. The multicopy nature of _ToxB_ and its homologs described herein, as well as the observed sequence variation of _Ptr toxb_, _Pb ToxB_, and MGG 09686.5 as compared to _Ptr ToxB_ (Figures 3.3A and 3.5), provides support for the premise that duplication and divergence shaped the evolutionary history of this HST gene. Furthermore, the low level of sequence identity remaining between _Ptr ToxB_ and MGG 09686.5 (Figure 3.5) suggests divergence from a common ancestor occurred early in evolutionary history. Indeed, Yoder (1998) cited the divergence of _ChPKSI_ and _MzmPKSI_, which retain 62% nucleotide identity, as support for their origination from a common ancestor as opposed to from horizontal gene transfer. Concordant evolution between _ToxB_ and our species phylogeny would provide additional support for the origination of this gene from a common ancestor. For example, congruence between a trypsin phylogeny and the organismal phylogeny for fungi was interpreted to suggest an evolutionary pathway in which trypsin divergence largely reflects the speciation of fungal lineages and its absence in specific fungi is thus predicted to be due to gene loss (Hu and St. Leger 2004). Toward this end, additional _ToxB_ homologs need to be isolated and sequenced for inclusion in a _ToxB_ gene genealogy for comparison to our species phylogeny.

The maintenance of _ToxB_-like sequences in a range of fungal lineages raises the question as to what selective advantage _ToxB_ confers to organisms in which it exists. For _P. tritici-repentis_, _Ptr ToxB_ confers pathogenicity/virulence on _Ptr ToxB_-sensitive wheat. The prevalence of _ToxB_-like sequences in plant pathogenic fungi, most of which infect grass hosts and thus may have evolved under similar selection pressures, highlights the possibility that _ToxB_ could specifically function in plant host-pathogen interactions. That _ToxB_-like sequences are present as multiple copies in these other species (Figure 3.2, Figure 3.4B-D), as is the case for _ToxB_ in pathogenic _P. tritici-repentis_ (Figure 3.2),
suggests ToxB homologs could be acting as HSTs. Alternatively, it is also feasible that ToxB serves as a general virulence factor for these fungi, as was reported for the acyl-AMP ligase-like enzyme CPS1 (Lu et al. 2003) and the nonribosomal peptide synthase NPS6 (Lee et al. 2005), which like ToxB, are distributed across ascomycetes. However, it could also be that the ToxB progenitor gene was recruited from primary metabolism for a new function (Hartmann et al. 2005), here pathogenicity, and that other fungi maintain yet to be determined functions. Finally, it is also possible that these sequences are historical remnants of ToxB, having succumbed to a fate as pseudogenes (Balakirev and Ayala 2003), although the discovery of intact homologs argues for the maintenance of a functional product. Although the multicopy nature of ToxB presents certain challenges, the availability of transformation, targeted gene disruption, and RNA silencing technologies for use in fungi (Idnurm and Howlett 2001, Michielse et al. 2005, Mullins and Kang 2001, Nakayashiki 2005, Valent and Chumley 1991, Wendland 2003, Xu and Xue 2002) will facilitate elucidation of ToxB function in these other fungal species. Functional characterization, in addition to a more robust sampling, promises to illuminate the evolutionary history of ToxB.

In contrast to the broad distribution of ToxB, ToxA is distributed in accordance with what is typical for an HST: thus far it has been shown to be present only in Ptr ToxA-producing, pathogenic isolates of P. tritici-repentis. ToxA was not detected by multiplex PCR or Southern analyses for any isolate used in this study (data not shown). However, a BLAST similarity search of available fungal genomes (www.broad.mit.edu/annotation/fungi) produced one hit in the genome of Stagonospora nodorum, causal agent of stagonospora nodorum blotch of wheat (Solomon et al. 2006). Hypothetical protein SNU16571.1 is 97% identical and 99% similar to Ptr ToxA. Comparison of the ORF nucleotide sequence reveals 98% identity between the two species. Friesen and colleagues (personal comm.) have subsequently identified a population of ToxA-like sequences required for disease, designated SnToxA, from numerous S. nodorum isolates. Based on the presence of sequence variation between various SnToxA sequences, and the lack thereof for ToxA, they hypothesize that SnToxA
was recently transferred to *P. tritici-repentis* from *S. nodorum* via horizontal gene transfer, possibly via anastomosis bridges between conidia. Similar to those described for *Colletotrichum* (Roca et al. 2003, Roca et al. 2004) and *Neurospora* (Roca et al. 2005), we have observed anastomosis bridges between conidia for both *P. tritici-repentis* and *P. bromi* (unpublished data). Nuclear exchange between conidia across these bridges could provide a mechanism for horizontal gene transfer, although this has yet to be experimentally demonstrated. Although both have converged on a similar fate as a HST, the evolutionary histories of *ToxA* and *ToxB* are distinct. Thus, *P. tritici-repentis* provides a model system with which to simultaneously investigate various mechanisms of HST evolution and the evolution of virulence.
Chapter 4

*Is PbToxB, a homolog of the host-selective toxin ToxB from *Pyrenophora tritici-repentis*,
at work in the *P. bromi*-bromeegrass interaction?*

Rachael M. Andrie and Lynda M. Ciuffetti

Prepared for Submission
Abstract

Ptr ToxB is one of multiple host-selective toxins produced by *Pyrenophora tritici-repentis*, causal agent of the disease tan spot of wheat. *P. bromi*, causal agent of brown spot of bromegrass, is one of several ascomycetes found to contain ToxB-like sequences. Due to the relatedness of *P. tritici-repentis* and *P. bromi*, and that of their grass hosts, we investigated if the ToxB homologs in *P. bromi*, designated *PbToxB*, act as HSTs involved in its disease interaction with bromegrass. To assess the role of various *Pb ToxB* proteins in the interaction between *P. bromi* and bromegrass, the corresponding *PbToxB* loci were heterologously expressed in *Pichia pastoris* as was done for the characterization of *ToxB* as an HST. Although *Pb ToxB* presumably plays a role in *P. bromi* biology as suggested by evidence of *PbToxB* transcription in culture, it does not act as an HST for *P. bromi* on bromegrass as shown by a lack of activity upon infiltration of heterologously-expressed *Pb ToxB* proteins. Despite that wheat is resistant to *P. bromi*, infiltration of various *Pb ToxB* proteins into wheat resulted in a range of chlorotic symptoms. A possible biological significance for this phenotype was supported by the expression of *PbToxB* transcript in *P. bromi*-inoculated wheat as determined by *in planta* reverse-transcription polymerase chain reaction. Thus, it is possible that *Pb ToxB* plays a role in establishment of an interaction between *P. bromi* on wheat. Alternatively, homologs of *ToxB* may represent evolutionary remnants of an "arms race" between ascomycetes and their grass hosts.

Introduction

Ptr ToxB (Orolaza et al. 1995, Strelkov et al. 1999) is one of multiple host-selective toxins (HSTs) produced by *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat (Ciuffetti and Tuori 1999, De Wolf et al. 1998, Strelkov and Lamari 2003). This proteinaceous HST is encoded by the ToxB gene (Martinez et al. 2001) and induces chlorosis on the sensitive wheat differentials Katepwa and 6B662 (Lamari et al. 1995). *P. tritici-repentis* races 5 (Lamari et al. 1995), 6 (Strelkov et al. 2002), 7 and 8 (Lamari et al. 2003), all of which produce *Ptr ToxB* and are pathogenic on *Ptr ToxB*-sensitive wheat,
each contain multiple copies of ToxB. Additionally, although they do not produce Ptr ToxB, nor are they pathogenic on Ptr ToxB-sensitive wheat, P. tritici-repentis races 3 and 4 each contain single-copy homologs of ToxB. Although the sequence of the ToxB homolog in race 3 is nearly identical to the ToxB open reading frame (ORF) except for the first six nucleotides, the upstream sequence is unique and potentially contains an alternate ORF start site (Strelkov et al. 2006). The ToxB homologs thus far identified in nonpathogenic P. tritici-repentis race 4 isolates are 86% identical to ToxB and have one additional codon in their ORFs (Martinez et al. 2004, Strelkov et al. 2006). The ToxB homolog from the race 4 isolate SD20 was named toxb due to its lack of an obvious function in pathogenicity in comparison to ToxB (Martinez et al. 2004). Additionally, evidence indicates differences in transcriptional regulation for the ToxB homologs in races 3 and 4 (Martinez et al. 2004, Strelkov et al. 2006). Other non-Ptr ToxB-producing races of P. tritici-repentis, including races 1, 2, 9, and 10, lack ToxB homologs (Chapter 2, Martinez et al. 2004), however produce other toxins (Chapter 2, Ciuffetti and Tuori 1999, De Wolf et al. 1998, Strelkov and Lamari 2003).

The distribution of ToxB-like sequences has also been shown to extend to other fungi (Chapter 3). The first ToxB homologs identified beyond the isolates of P. tritici-repentis were the PbToxB loci from P. bromi (Chapter 3), the species most closely related to P. tritici-repentis (Zhang and Berbee 2001). P. bromi is the causal agent of brownspot of bromegrass (Bromus inermis) (Chamberlain and Allison 1945), the grass species most closely related to wheat (Barker et al. 2001, Hsiao et al. 1994). Like ToxB in P. tritici-repentis, PbToxB in P. bromi is found both as a single copy and in multiple copies. Comparison of the PbToxB sequences to ToxB and toxb reveals a triangle of identity between these loci (Chapter 3). Although slight variation exists between the different PbToxB loci, PbToxB is on average 87% identical to both ToxB and toxb at the nucleotide level, and at the deduced amino acid level, Pb ToxB is on average 81% identical to Ptr ToxB and Ptr toxb. Additionally, as is observed for toxb, all PbToxB sequences have an additional codon in their ORF. ToxB-like sequences were demonstrated to be present in other fungi in the Pleosporaceae via Southern analysis, including throughout members of
the *Pyrenophora*, and in the closely-related genera *Cochliobolus* and *Alternaria* (Chapter 3). Although *P. tritici-repentis* belongs to the class Dothideomycetes (Eriksson 2005), a distant homolog was identified in *Magnaporthe grisea* (Chapter 3), a member of the Sordariomycetes (Eriksson 2005). The broad distribution of ToxB is unique for a HST gene, which in general are found exclusively in pathogenic isolates of a single species with no corresponding allele or locus in nonpathogenic isolates. This raises the question as to what function(s) these ToxB-like sequences provide for other fungi in which they are found.

Due to the relatedness of *P. bromi* and *P. tritici-repentis* and their grass hosts, it is possible that PbToxB encodes for a HST involved in the interaction between *P. bromi* and bromegrass. Indeed, due to the presence of chlorotic halos beyond the boundaries of fungal hyphae, a phytotoxin has been implicated in brownspot of bromegrass caused by *P. bromi* (Sherwood 1996). To investigate whether PbToxB encodes for a HST, we employed a similar approach previously developed for the characterization of the role of ToxB in the interaction between *P. tritici-repentis* and wheat. Analysis of heterologously-expressed Ptr ToxB confirmed that ToxB encodes for a HST (Martinez et al. 2001). Various PbToxB loci were heterologously expressed in *Pichia pastoris*, followed by assessment of their toxicity on bromegrass. Although PbToxB does not encode for an HST in the interaction between *P. bromi* and bromegrass, we revealed evidence that PbToxB may provide a function for *P. bromi* on wheat.

**Materials and Methods**

**Fungal isolates and plant material**

The following isolates were used in this study. The *P. bromi* isolates SM101, SM106, and Bf-1 (Chapter 3), and the *P. tritici-repentis* race 5 isolate DW7 (Ali et al. 1999) were obtained from L. Francl and S. Ali (North Dakota State University, Fargo). The *P. tritici-repentis* race 4 isolate SD20 (Martinez et al. 2004) was obtained from G. Buchenau (South Dakota State University, Brookings). Culture conditions are published elsewhere (Chapter 2). The smooth bromegrass varieties chosen for this study included
the line PL-BDR1 (Berg et al. 1989) and the cultivar Baylor, both obtained from the U.S. National Plant Germplasm System, USDA, ARS, Western Regional PI Station (Pullman, WA), as well as a commercial cultivar obtained from Outsidepride.com, Inc. (Independence, OR). For wheat, the Ptr ToxB-sensitive cultivar Katepwa and line 6B662 (Lamari et al. 1995), and the resistant cultivar Auburn (Chapter 2, Ciuffetti and Tuori 1999, Tomas and Bockus 1987, Tomas et al. 1990) were used. Prior to inoculation or infiltration, smooth bromegrass was grown in a peat-vermiculite (1:1) mix for four to five weeks and wheat was grown in soil for two to three weeks under diurnal conditions of light for 16 h at 23°C and darkness for 8 h at 19°C.

**Fungal inoculation of plant hosts**

Conidial concentrations for inoculation of smooth bromegrass and wheat were $1.25 \times 10^3$ (Zeiders and Sherwood 1986) and $3 \times 10^3$ (Lamari and Bernier 1989a) conidia/ml, respectively, regardless of whether *P. bromi* or *P. tritici-repentis* were used. Plants were sprayed to drip stage, incubated overnight in a dark dew chamber at ~20°C to 22°C, and returned to diurnal conditions. Disease development was subsequently monitored, and secondary or tertiary leaves for wheat and bromegrass, respectively, were harvested at 5 to 8 days post inoculation (dpi) to document the infection phenotype. For RNA extractions, leaves of wheat cultivar Katepwa and line 6B662, inoculated with either *P. tritici-repentis* isolate DW7 or *P. bromi* isolates SM101 and SM106, were harvested 2 dpi. Inoculations were repeated at least 2 to 4 times with similar results.

**RNA extraction and DNase treatment**

RNA was extracted from fungal tissue grown in modified Fries medium (Tomas and Bockus 1987) at 25°C under constant illumination for 10 days, or from fungal infected leaf tissue 2 dpi, per a modification of the manufacturer’s instructions for the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Two hundred milligrams of either type of tissue were ground in liquid nitrogen followed by the immediate addition of 900 μl RLT lysis buffer with thorough mixing. Following a 2 min incubation at 56°C with
constant mixing, samples were vortexed for 30 s. Seven hundred fifty microliters were transferred to a QIAshredder mini spin column for homogenization, after which 450 µl of the flow-through were processed as described by manufacturer's instructions, including the optional on-column DNase digestion with the RNase-Free DNase set (Qiagen). RNA samples were quantified spectrophotometrically and their quality verified via agarose gel electrophoresis. Further DNA removal required rigorous DNase treatment with TURBO DNase as described for the TURBO DNA-free kit (Ambion, Austin, TX). RNA samples were diluted to 9 µg/40 µl prior to treatment, and TURBO DNase was applied twice followed by a 30 min incubation at 37°C each time. Prior to subsequent analyses, all samples were screened by polymerase chain reaction (PCR) analysis with ToxB-specific primers for contamination with genomic DNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For each sample, first-strand cDNA was synthesized from 1.5 µg total RNA as described in the manufacturer's instructions for SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, CA). Following treatment with RNase, two cDNA reactions per sample were subsequently combined and concentrated on a MinElute spin column (Qiagen), which also served to remove the poly-dT primer as was done by Martinez and colleagues (2004). RT-PCR reactions (50 µl) consisted of 1× Taq polymerase buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0), 200 µM each dNTP, 200 nM each primer, 2.5 U Taq polymerase (MBI Fermentas, Hanover, MD), and either 10% of the combined cDNA reaction or 300 ng of the original RNA sample, which corresponds to the total amount of RNA found in 10% of the combined cDNA reaction. Primers TB7F (Martinez et al. 2001) and TB59R (Martinez et al. 2004) were used for the detection of both ToxB and PbToxB transcripts. Amplification was carried out as follows: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 35s, 72°C for 55s; 1 cycle of 72°C for 7 min. As degraded RNA migrates similarly to the ToxB and PbToxB transcripts, PCR reactions containing RNA were treated with RNase following amplification. For each sample, two cDNA and two RNA reactions were combined and concentrated on
MinElute spin columns (Qiagen) for visualization via standard agarose gel electrophoresis. Amplification product sizes were determined via comparison to the MassRuler™ DNA Ladder, Mix, ready-to-use (MBI Fermentas, Hanover, MD). Amplification product identity was confirmed via sequence analysis (Central Services Laboratory [CSL], Center for Genome Research and Biocomputing [CGRB], Oregon State University [OSU], Corvallis, OR). For *P. bromi*, in culture and *in planta* RT-PCR was repeated twice and three times, respectively, with similar results. For *P. tritici-repentis*, in culture RT-PCR was repeated four times, and *in planta* RT-PCR was repeated two and three times for Katepwa and 6B662, respectively.

**Heterologous expression in *Pichia pastoris***

Constructs for heterologous expression in *P. pastoris* were assembled in accordance with the instructions for the EasySelect™ *Pichia* Expression kit (Invitrogen). In conjunction with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), primer PbTB1F (5'-TAGCTCGAGAAAAGAAACTGCATCGCCAATATC-3'), which introduced a 5' XhoI site, and primer TB16R (Martinez et al 2001), which introduced a 3' NotI site, were used to amplify the coding sequence for each mature protein. The reaction mixture was composed of 1× High Fidelity PCR Buffer (18 mM ammonium sulfate, 60 mM Tris-SO₄, pH 8.9), 2 mM MgSO₄, 200 µM each dNTP, 200 nM each primer, 1 U Platinum *Taq* High Fidelity (Invitrogen), and 10 ng template DNA in 25 µl. Amplification was carried out as follows: 1 cycle of 94°C for 1 min; 31 cycles of 94°C for 20 s, 55°C for 20 s, 68°C for 2 min 20 s; 1 cycle of 68°C for 7 min. Gel-purified (Qiagen) amplification products were digested with *XhoI* and *NotI* and cloned flush with the Kex2 cleavage site of expression vector pPICZαB, followed by transformation into *Escherichia coli* XL-Blue competent cells. Positive clones for each were confirmed via sequence analysis (CSL, CGRB, OSU, Corvallis, OR).

Constructs were linearized with *SacI* for transformation into *P. pastoris* X-33 competent cells as specified in the instructions for the *Pichia* EasyComp™ kit (Invitrogen). Positive clones were chosen for heterologous expression in buffered
minimal methanol media as specified by the instructions for the EasySelect™ Pichia Expression kit (Invitrogen). Each culture filtrate was lyophilized, resuspended in water to approximately 1/10th the original volume, and desalted on a PD-10 Desalting column per manufacturer’s instructions (GE Healthcare, Piscataway, NJ). For use as a control, culture filtrate from P. pastoris X-33 transformed with the pPICZαB vector alone was processed as described above.

Detection and quantitation of heterologously-expressed proteins

Protein samples were fractionated by 13% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in conjunction with the buffer system of Fling and Gregerson (1986), detected with the SilverSNAP® Stain Kit II (Pierce Biotechnology, Inc., Rockford, IL), and compared to the MultiMark® Multi-Colored Standard (Invitrogen). For heterologous protein quantitation, the silver-stained SDS-PAGE gel was documented via digital scanning for subsequent densiometric analysis with ImageQuaNT 5.0 as described by manufacturer’s instructions (Molecular Dynamics Inc, Sunnyvale, CA). ImageQuaNT 5.0 compares the relative intensity of bands, as measured in pixel volume, on the same gel. The parameters used included volume integration, which evaluates the relative density of bands in different lanes at the same distance from the top of the lane, and object average background correction, which subtracts the average pixel value of an identically-sized null area of the gel from each sample. Band volumes for a set of four standards generated from a purifiedPtr ToxB stock of known concentration were used to calculate a standard curve (y = 1.44.51x – 10798; R² = 0.9428) from which the amount of protein (total ng) in unknown bands could be extrapolated. Band volumes of two replicates for each sample were averaged, followed by comparison to the standard curve to determine the relative amount of heterologously-expressed protein present. Sample concentrations were subsequently normalized to 9.5 ng/µl, 19 ng/µl, and 38 ng/µl for infiltration.
Western blot analysis of heterologously-expressed proteins

Protein samples were fractionated as described above, followed by gel-equilibration in transfer buffer (25 mM Tris Base, 193 mM glycine, 20% methanol) and dry transfer of proteins to GE Nitrocellulose Pure Transfer Membrane (GE Osmonics, Minnetonka, MN). Resultant blots were blocked overnight at 4°C in 1× TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCL, pH 8.0), 3% nonfat dried milk. Blots were then washed in 1× TBST, followed by incubation for 1 h in anti-Ptr ToxB antisera (Andrie et al. 2005) diluted 1:2,000, in 1× TBST, 1% BSA fraction V. Blots were again washed, followed by incubation for 0.5 h in anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:8,000 in 1× TBST. Following a final wash, blots were developed via chemiluminescence in accordance with manufacturer’s instructions for the SuperSignal® West Dura Extended Duration Substrate (Pierce). Western analysis was repeated with at least 2 sets of independently-expressed proteins.

Bioassay of heterologously-expressed proteins

A modified Hagborg device (Hagborg 1970) was used to infiltrate heterologously-expressed proteins into secondary and tertiary leaves of wheat and bromegrass, respectively. Three leaves were infiltrated per treatment, after which plants were returned to the growth chamber. Leaves were harvested at either day 5 or day 7 postinfiltration for wheat and bromegrass, respectively, and the extent of chlorosis documented via digital scanning. Infiltrations were repeated with 2 to 4 sets of independently-expressed proteins.

Results

In culture expression analysis of PbToxB

We first confirmed that the PbToxB gene is actively transcribed. Conditions under which P. tritici-repentis race 5 isolate DW7 is known to produce ToxB mRNA (Martinez et al. 2004) were used to grow P. bromi for RNA extraction. To maximize
detection of potentially rare \textit{PbToxB} transcripts, the amplification products from two reverse transcriptase-polymerase chain reactions (RT-PCR) were combined and concentrated for agarose gel electrophoresis. Results of RT-PCR analysis showed that \textit{PbToxB} from \textit{P. bromi} is actively transcribed in culture (Figure 4.1A). However, a comparison of the RT-PCR results for \textit{P. bromi} isolates SM101 and SM106 (Figure 4.1A) with the RT-PCR results for \textit{P. tritici-repentis} isolate DW7 (Figure 4.1B), suggest that the level of \textit{PbToxB} expression is less than that for \textit{ToxB} under the same conditions. For all samples the same amount of starting RNA was used for first-strand cDNA synthesis and the same amount of cDNA was used in subsequent PCR reactions. Additionally, the same amount of DNA mass ladder was loaded on each gel. Thus, the intensity differences observed in Figure 4.1 reflect quantitative differences between \textit{ToxB} and \textit{PbToxB} transcript levels. An RNA control was included to rule out contamination of genomic DNA in cDNA samples as the source of amplification signal. Similar results were obtained for \textit{P. bromi} isolate Bf-1 (data not shown).

**Heterologous expression of chosen \textit{PbToxB} loci**

To determine if the \textit{ToxB} gene from \textit{P. tritici-repentis} race 5 isolates encoded for a HST, Martinez and colleagues (2001) infiltrated heterologously-produced \textit{Ptr ToxB} into the standard set of wheat differentials (Lamari and Bernier 1989a, Lamari et al. 1995). The observed correlation between susceptibility to race 5 and sensitivity to \textit{Ptr ToxB} confirmed that \textit{ToxB} encoded for a HST. We employed a similar approach to determine if \textit{PbToxB} functions as a HST in the \textit{P. bromi}-bromegrass interaction. To visually represent the relationships among the eight characterized \textit{PbToxB} (Chapter 3), an unrooted neighbor-joining tree (Saitou and Nei 1987) of corresponding \textit{Pb ToxB} deduced amino acid sequences was constructed in BioEdit (Hall 1999) (Neighbor-Joining/UPGMA method version 3.6a2.1) (Figure 4.2A). At least one protein from each of the three observed \textit{Pb ToxB} clades from the resultant tree was chosen for heterologous expression in \textit{Pichia pastoris}. Two additional \textit{Pb ToxB} proteins were also chosen for expression, such that the protein products encoded by all of the \textit{PbToxB} loci found in the
Figure 4.1. *PbToxB* from *Pyrenophora bromi* is actively transcribed in culture, though at lower levels than that of *ToxB* from *P. tritici-repentis*.

A) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *PbToxB* expression from *P. bromi* isolates SM101 and SM106. B) RT-PCR analysis of *ToxB* expression from *P. tritici-repentis* race 5 isolate DW7. Fifteen microliters of DNA mass ladder were loaded on each agarose gel. *Pb* = *P. bromi*, *Ptr* = *P. tritici-repentis*, Marker = DNA mass ladder, with band sizes given in base pairs.
Figure 4.2. Heterologous expression of PbToxB loci.

A) A neighbor-joining tree based on deduced Pb ToxB amino acid sequences was used to choose a subset of PbToxB loci (designated by *) for heterologous expression. B) Silver-stained SDS-polyacrylamide gel of heterologously-expressed Pb ToxB, Ptr toxb, and Ptr ToxB proteins for densiometric quantitation via comparison to a set of purified Ptr ToxB standards (Std), including 75 ng, 150 ng, 225 ng, and 350 ng (from left to right). Pb ToxB proteins are represented by their associated locus number, including Pb ToxB5-1 and Pb ToxB5-2 from P. bromi isolate SM106; Pb ToxB6-1 and Pb ToxB6-2 from P. bromi isolate SM101; and, Pb ToxB7-2 from P. bromi isolate Bf-1.
P. bromi isolates SM101, SM106, and Bf-1 were represented. Thus, the five PbToxB loci chosen for heterologous expression included: PbToxB5-1 and PbToxB5-2 from P. bromi isolate SM106, PbToxB6-1 and PbToxB6-2 from P. bromi isolate SM101, and PbToxB7-1 from Bf-1. As shown in Figure 4.2A, PbToxB7-2 and PbToxB2 are identical to PbToxB7-1, and PbToxB1 is identical to PbToxB5-1, thus results obtained from analysis of heterologously-expressed Pb ToxB7-1 and Pb ToxB5-1 should be applicable to these other proteins.

Heterologous expression of PbToxB in P. pastoris relied on a signal sequence to target expressed proteins to the secretory pathway. The α-factor signal sequence from Saccharomyces cerevisiae in vector pPICZαB was chosen due to the results of a preliminary screen that revealed expression from the α-factor signal sequence to be superior to that from the native signal sequence (data not shown). Five-day-old culture filtrates for each clone were analyzed via 13% SDS-polyacrylamide gel electrophoresis to confirm heterologous expression (Figure 4.2B). Heterologously-expressed Pb ToxB proteins were of similar size to Ptr ToxB, which is 6.6 kDa (Martinez et al. 2001, Strelkov et al. 1999). All heterologously-expressed samples shared a conglomerate of large proteins with the pPICZαB vector control (data not shown). Densitometry of a digital SDS-PAGE image was used to estimate the amount of the heterologously-expressed protein of interest in each culture filtrate. Average band intensities of each Pb ToxB protein in duplicate were compared to a standard curve calculated from the band intensities for a set of purified Pb ToxB standards, including 75, 150, 225, and 350 ng (Figure 4.2B). For leaf infiltration bioassays, heterologous protein concentrations were normalized to 9.5 ng/µl, 19 ng/µl, and 38 ng/µl. The concentrations 9.5 ng/µl and 20 ng/µl were chosen based on known physiologically relevant concentrations of native Ptr ToxB (Strelkov et al. 1999) and of heterologously-expressed Ptr ToxB (Martinez et al. 2001), respectively. The vector only control was applied without dilution relative to the other samples.
Western analysis of Pb ToxB as compared to Ptr ToxB and Ptr toxb

Western analysis was performed to determine the affinity of the anti-Ptr ToxB antibody for the various Pb ToxB proteins (Figure 4.3). Initially, western analysis was performed with equal amounts of each protein, however, the signal for Ptr ToxB from recognition by the anti-Ptr ToxB antibody overwhelmed the signal of this antibody for all of the other proteins (data not shown). Thus, we subsequently loaded 10-fold more of each Pb ToxB and of Ptr toxb as compared to Ptr ToxB. Although the anti-Ptr ToxB antibody recognized Pb ToxB and Ptr toxb, it did so with at least 10-fold less affinity than for Ptr ToxB, as shown by a similar to less intense signal upon antibody recognition even though 10-fold more Pb ToxB and Ptr toxb (750 ng) was loaded on the gel as compared to Ptr ToxB (75 ng) (Figure 4.3). Recognition of the various Pb ToxB proteins and of Ptr toxb by the anti-Ptr ToxB antibody confirms that these proteins are Ptr ToxB-like, however affinity differences reflect their sequence divergence.

Representative inoculations of P. bromi and P. tritici-repentis on bromegrass and wheat

Figure 4.4A shows representative reactions of bromegrass and Ptr ToxB-sensitive wheat to inoculation by P. bromi and P. tritici-repentis races 4 and 5. A PbToxB-containing P. bromi isolate causes disease on its host, bromegrass, although exhibits a resistant reaction on wheat. ToxB-containing P. tritici-repentis race 5 isolate DW7 exhibits the inverse response, a disease phenotype on its host, wheat, and a resistant reaction on bromegrass. Finally, toxb-containing P. tritici-repentis race 4 isolate SD20 displays a resistant reaction on both bromegrass and wheat.

Infiltration bioassays of heterologously-expressed Pb ToxB into bromegrass

If Pb ToxB functions as a HST in the interaction between P. bromi and bromegrass, infiltration of Pb ToxB proteins alone is expected to reproduce disease symptoms on correspondingly susceptible host plants. Infiltration of heterologously-expressed Pb ToxB proteins into three bromegrass cultivars, including Baylor, PL-BDR1, and a commercial variety, at the highest concentration chosen for infiltration, 38 ng/µl,
Figure 4.3. Western analysis of heterologously-expressed Pb ToxB proteins as compared to Ptr ToxB and Ptr toxb.

Seven hundred fifty nanograms of each Pb ToxB protein and of Ptr toxb, and 75 ng of Ptr ToxB, were separated by SDS-polyacrylamide electrophoresis followed by western blot analysis with anti-Ptr ToxB antibodies. As in Figure 4.2, Pb ToxB proteins are represented by their associated locus number. Control = culture filtrate from a *Pichia pastoris* clone transformed with pPICZaB vector alone.
Figure 4.4. Pb ToxB lacks phytotoxic activity on smooth bromegrass susceptible to *Pyrenophora bromi*, though elicits chlorosis on Ptr ToxB-sensitive wheat.

A) Typical reactions of bromegrass and Ptr ToxB-sensitive wheat to inoculation with *P. bromi*, *P. triticic-repentis* race 5, and *P. triticic-repentis* race 4. B) Infiltration of heterologously-expressed Pb ToxB, Ptr ToxB, and Ptr toxb into bromegrass. Infiltrations were done at a concentration of 38 ng/μl. C) Infiltration of heterologously-expressed Pb ToxB, Ptr ToxB, and Ptr toxb into Ptr ToxB-sensitive wheat cultivar 6B662. Infiltrations were done at a range of concentrations, including: 9.5 ng/μl, 19 ng/μl, and 38 ng/μl. As in Figure 4.2, Pb ToxB proteins are represented by their associated locus number. *Pb-PbToxB* = inoculated with a *PbToxB*-containing *P. bromi* isolate; *Ptr-ToxB* = inoculated with a *ToxB*-containing *P. triticic-repentis* race 5 isolate; *Ptr-toxb* = inoculated with a *toxb*-containing *P. triticic-repentis* race 4 isolate. Control = culture filtrate from a *Pichia pastoris* clone transformed with pPICZaB vector alone.
failed to elicit symptoms on bromegrass. A representative leaf for each Pb ToxB protein is shown in Figure 4.4B. As the P. bromi isolates SM101 and SM106 contain two different PbToxB loci, it is possible that the formation of a Pb ToxB heterodimer is necessary for toxicity on bromegrass. To investigate this possibility, both variants from SM101 (Pb ToxB6-1 and 6-2) or SM106 (Pb ToxB5-1 and 5-2) were mixed and co-infiltrated into bromegrass; however, there still was no effect of Pb ToxB on bromegrass (data not shown). Ptr ToxB, Ptr toxb, and the vector only control also did not cause any reaction on bromegrass (Figure 4.4B). Our results indicate that Pb ToxB does not function as a HST in brownspot of bromegrass at least on tested bromegrass; yet it is expressed, thus presumably plays a role in P. bromi biology.

Infiltration bioassays of heterologously-expressed Pb ToxB into wheat

To investigate whether the product of PbToxB possibly provides a function for P. bromi on wheat, heterologously-expressed Pb ToxB proteins were infiltrated into Ptr ToxB-sensitive wheat, in comparison to Ptr ToxB, Ptr toxb, and a vector only control (results for wheat line 6B662 are shown in Figure 4.4C). As expected, infiltration of Ptr ToxB resulted in characteristic chlorosis, the intensity of which increased with increasing concentrations of toxin applied. Additionally, no symptoms were elicited from infiltration of Ptr toxb or the control. However, a range of symptoms were elicited on wheat upon infiltration of the various Pb ToxB proteins. Pb ToxB7-2 from P. bromi isolate Bf-1 gave no reaction at any concentration. PbToxB5-1 from SM106 only caused chlorosis at the highest concentration, 38 ng/µl. PbToxB5-2 from SM106 and PbToxB6-2 from SM101 gave hints of chlorosis at 9.5 ng/µl, which intensified at the higher concentrations of 19 ng/µl and 38 ng/µl, but never reached levels of chlorosis caused by Ptr ToxB. Finally, PbToxB7-1 from isolate SM101 acted similarly to Ptr ToxB at all three concentrations. Similar results were also obtained for infiltration of all heterologous proteins on the Ptr ToxB-sensitive wheat cultivar Katepwa (data not shown). Additionally, none of the Pb ToxB proteins elicited chlorosis on insensitive cultivar Auburn (data not shown), thus the observed activity is host-specific like that of
Ptr ToxB. Despite a resistant reaction upon inoculation of Ptr ToxB-sensitive wheat with *P. bromi* (Figure 4.4A), various Pb ToxB proteins are capable of eliciting chlorosis (Figure 4.4C).

**Analysis of PbToxB expression by Pyrenophora bromi on wheat**

Although heterologously-expressed Pb ToxB proteins elicited symptoms on wheat upon infiltration, a biological basis had yet to be established. RT-PCR was used to determine whether *PbToxB* is expressed in *P. bromi*-inoculated wheat (Figure 4.5). We pursued *P. bromi* isolates SM101 and SM106, because their Pb ToxB variants elicited chlorosis on Ptr ToxB-sensitive wheat. Two days post inoculation (dpi) was chosen for RNA extraction of fungal-inoculated wheat for the following reasons: 1. it was previously shown that infection of susceptible and resistant plants by *P. tritici-repentis* proceeds similarly up to 48 hours, after which either disease progresses or plant defense responses halt pathogen ingress, respectively (Dushnicky et al. 1998); 2. infection phenotypes on wheat for both *P. tritici-repentis* race 5 and *P. bromi* look similar up to this point (data not shown), although by 5 to 8 dpi, leaves infected with *P. tritici-repentis* are diseased, whereas those infected with *P. bromi* exhibit a resistance response (Figure 4.4A); and 3. surface conidia, 95% of which have germinated after 6 h of constant leaf wetness on both resistant and susceptible cultivars (Larez et al. 1986), are likely dehydrated (De Wolf et al. 1998), and thus their associated RNA degraded.

To increase sensitivity of rare transcript detection, especially due to the dilution of fungal transcripts among a majority of plant transcripts, two PCR reactions were combined for agarose gel electrophoresis as was done for RT-PCR in culture. RT-PCR results of fungal-infected wheat revealed that *PbToxB* is expressed in wheat for both *P. bromi* isolates SM101 and SM106 (Figure 4.5A). Although the exact amount of fungal RNA in each sample could not be exactly quantified due to presence of a mixture of both plant and fungal RNA, *in planta* levels of *PbToxB* transcription for *P. bromi* in Ptr ToxB-sensitive wheat (Figure 4.5A) were obviously much lower than that of *ToxB* for pathogenic *P. tritici-repentis* isolate DW7 inPtr ToxB-sensitive wheat (Figure 4.5B).
Figure 4.5. *PbToxB* from *Pyrenophora bromi* is actively transcribed in *Ptr* ToxB-sensitive wheat, though at levels less than that of ToxB from *P. tritici-repentis*.

A) *In planta* reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *PbToxB* expression in *Ptr* ToxB-sensitive wheat cultivar Katepwa and line 6B662 inoculated with *P. bromi* isolates SM101 and SM106. 

B) *In planta* RT-PCR analysis of ToxB expression in Katepwa and 6B662 inoculated with *P. tritici-repentis* isolate DW7. 

C) *In planta* RT-PCR analysis of noninoculated wheat controls. Fifteen microliters of DNA mass ladder were loaded on each agarose gel. *Pb* = *P. bromi*, *Ptr* = *P. tritici-repentis*, Kat = Katepwa, Marker = DNA mass ladder, with band sizes as in Figure 4.1.
RNA only (Figure 4.5A-C) and noninoculated (Figure 4.5C) controls were free of contaminating genomic DNA. That PbToxB is expressed by *P. bromi* on wheat at 2 dpi suggests a possible function of the Pb ToxB protein in the interaction between *P. bromi* and wheat.

**Discussion**

In order to be designated as a HST a molecule must be: 1. toxic only to hosts of the fungus that produce the toxin; 2. produced coincident with disease; and, 3. a primary determinant of pathogenicity or virulence (Scheffer and Livingston 1984, Yoder 1980). In an effort to determine if PbToxB encodes for a HST in the interaction between *P. bromi* and bromegrass, we first investigated whether the product of this gene is toxic to sensitive hosts. Following confirmation that PbToxB is actively transcribed (Figure 4.1), and thus is not a genomic relic, various PbToxB loci were heterologously expressed in *P. pastoris* (Figure 4.2). However, all heterologously-expressed Pb ToxB proteins lacked activity on bromegrass upon infiltration (Figure 4.4A), thus evidence suggests Pb ToxB does not function as a HST in this pathosystem, at least on tested bromegrass.

That Pb ToxB does not function as a HST in the interaction between *P. bromi* and bromegrass does not preclude that a phytotoxin is involved in this disease. An assortment of HSTs is produced by various members of the *Pleosporaceae* (Walton 1996, Wolpert et al. 2002), the family to which *P. bromi* belongs (Eriksson 2005). In addition to the HSTs produced by *P. tritici-repentis*, a diversity of phytotoxic metabolites has been described for other grass pathogens of the genus *Pyrenophora*. Three toxins have been isolated from culture filtrates of *P. teres*, causal agent of net blotch of barely, two of which are secondary metabolites whose activity correlates with disease (Weiergang et al. 2002). *P. graminea*, causal agent of leaf stripe disease of barley, produces a high molecular weight glycoprotein in culture filtrates, the carbohydrate portion of which is involved in its interaction with barley (Haegi and Porta-Puglia 1995). Finally, *P. semeniperda* culture filtrates contain metabolites that are selectively toxic to susceptible hosts, although their nature remains to be characterized (Campbell et al. 2003). To
identify an alternate phytotoxic activity involved in the interaction between *P. bromi* and bromegrass, culture filtrates of *P. bromi* will have to be analyzed similarly to that done for the identification of other phytotoxins.

The expenditure of energy on *PbToxB* expression (Figure 4.1) suggests *PbToxB* provides a function in the biology of *P. bromi*. To investigate an alternate role of *PbToxB* for *P. bromi*, other than as a HST on bromegrass, we examined whether various *Pb ToxB* proteins are toxic to wheat. Heterologously-expressed *Pb ToxB* proteins were infiltrated at concentrations equivalent to those where *Ptr ToxB* is known to elicit chlorosis on sensitive wheat. As compared to the activity of heterologously-expressed *Ptr ToxB* infiltrated at the same concentrations, infiltration of various *Pb ToxB* proteins into *Ptr ToxB*-sensitive wheat resulted in a range of chlorotic symptoms, from no activity (e.g. *Pb ToxB7-2*) to activity similar to *Ptr ToxB* (e.g. *Pb ToxB6-1*) (Figure 4.4B), despite that *P. bromi* elicits a resistance response on wheat (Figure 4.4C). One to two amino acid differences between the various *Pb ToxB* proteins (Chapter 3) presumably account for observed differences in chlorotic symptoms elicited upon their infiltration. For those *Pb ToxB* proteins that elicited chlorosis, corresponding expression of *PbToxB* in *P. bromi*-inoculated wheat was subsequently confirmed. *In planta* RT-PCR analyses revealed that *PbToxB* is expressed at 2 dpi in wheat inoculated with *P. bromi* isolates SM101 and SM106 (Figure 4.5A), which suggests that the observed toxic activity on wheat resulting from infiltration of various *Pb ToxB* proteins could have biological significance.

One possible function for *Pb ToxB* could be to mediate a disease interaction between *P. bromi* and wheat, similar to the role of *Ptr ToxB* in the interaction between *P. tritici-repentis* and wheat. However, *P. bromi* induces a resistance response on wheat (Figure 4.4C). As suggested by quantitative expression differences between *PbToxB* and *ToxB* in culture by *P. bromi* and *P. tritici-repentis*, respectively (Figure 4.1), a potential explanation for this contradiction is that expression of *PbToxB* as compared to *ToxB* is too low to elicit disease. The noticeably lower *PbToxB* transcript levels produced on wheat at 2 dpi by *P. bromi* isolates SM101 and SM106 as compared to *ToxB* transcript
levels produced by pathogenic *P. tritici-repentis* race 5 isolate DW7 (Figure 4.5) supports this explanation. Two days post inoculation was shown to be the point at which interactions between *P. tritici-repentis* and susceptible and resistant wheat diverge, in that hyphae in susceptible plants continue to invade, whereas in resistant plants, hyphae are restricted to the site of infection by host resistance mechanisms (Dushnicky et al. 1998), presumably due to the production of an HST or the lack thereof. Thus, *PbToxB* transcript expression levels may be below those necessary to produce enough Pb ToxB protein to overcome the onset of plant resistance responses. Determination of whether low *PbToxB* transcript levels translate into correspondingly low Pb ToxB levels awaits the availability of Pb ToxB-specific antibodies, as the anti-Ptr ToxB antibody lacks the ability to specifically detect Pb ToxB proteins in culture filtrates above background potentially due to their sequence divergence (data not shown).

Due to their inability to detect Ptr ToxB in culture filtrates via anti-Ptr ToxB western analysis, Strelkov and colleagues (2002) invoked low levels of Ptr ToxB expression by *P. tritici-repentis* race 5 isolate 92-171R5 to explain its reduced lesion development on Ptr ToxB-sensitive wheat as compared to the highly virulent race 5 isolate Alg 3-24. Two possible explanations for the differences between these two race 5 isolates were presented: 1. the presence of different numbers of ToxB loci; or, 2. the differential transcriptional regulation of the various ToxB loci present (Strelkov et al. 2006). Although the ToxB ORFs were shown to be identical for all race 5 isolates, isolate 92-171R5 contains only two copies of ToxB as compared to the 8 to 10 copies found in Alg 3-24 (Strelkov et al. 2006). Although it is not yet known if all ToxB loci are actively transcribed, inoculation of nonpathogenic *P. tritici-repentis* race 4 transformants containing variable numbers of ToxB under the control of the same constitutive promoter revealed a positive correlation between ToxB copy number and chlorotic symptom development (Martinez and Ciuffetti, unpublished data). These results corroborate that fewer copies of ToxB could contribute to a reduction in disease severity, and suggest that the presence of multiple, active copies of ToxB in an isolate, or at least high Ptr ToxB production, are necessary to cause disease on Ptr ToxB-sensitive wheat. Thus, that *P. 
bromi isolates SM101 and SM106 each only have two copies of PbToxB (Chapter 3) provides a compelling explanation for the lack of symptomology on wheat caused by these P. bromi isolates despite the toxicity of the corresponding Pb ToxB proteins. As described for ToxB, to determine whether the presence of multiple copies of PbToxB in an isolate would be sufficient to cause disease on Ptr ToxB-sensitive wheat, the level of disease caused by P. bromi isolates with additional copies of PbToxB or nonpathogenic P. tritici-repentis race 4 transformants with variable numbers of PbToxB could be assessed.

Alternatively, Strelkov and Lamari (Strelkov and Lamari 2003) report that sequences upstream of the ToxB loci in P. tritici-repentis isolate 92-171R5 are substantially divergent from those upstream of the various ToxB loci in Alg 3-24, thus suggest that regulatory differences could also explain differences in Ptr ToxB production and disease severity caused by these isolates. Indeed, it was subsequently shown that transcription of ToxB in the less virulent race 5 isolate 92-171R5 is differentially regulated as compared to ToxB in the highly virulent isolate Alg 3-24 (Strelkov et al. 2006). ToxB in Alg 3-24 is expressed in both mycelia and conidia, whereas ToxB in 92-171R5 is expressed only in conidia. Thus, a reduction in disease caused by 92-171R5 as compared to Alg 3-24 could just as likely be due to expression differences as differences in ToxB copy number. Martinez and colleagues (2004) also hypothesized that sequence differences upstream of ToxB and toxb could account for observed differences in their transcriptional regulation. Although sequences upstream of the various PbToxB loci were quite similar to those upstream of ToxB, we identified the presence of variably-sized indel sequences upstream of the various PbToxB ORFs in a similar position as the distinct indel sequences upstream of toxb (Chapter 3). Thus, the inability of PbToxB-containing P. bromi isolates to overcome wheat defense responses and cause disease could also be explained by differential transcriptional regulation of the PbToxB loci as compared to ToxB.

Another possible function for the product of PbToxB is that it mediates a resistance response between P. bromi and wheat. An important component of disease
resistance in plants is the gene-for-gene interaction, in which the product of a plant resistance (R) gene recognizes the product of a pathogen avirulence (Avr) gene, and as a result elicits a myriad of defense responses to halt pathogen ingress (Dangl and Jones 2001). Coevolution of the gene-for-gene interaction has been described as an "arms race", or an ongoing dance of adaptation and counter-adaptation between the host and pathogen (Dawkins and Krebs 1979, Nimchuk et al. 2001, Stahl and Bishop 2000). It is hypothesized that host plants evolve recognition mechanisms for the detection of pathogen virulence factors, thus transforming them into avirulence determinants that betray the pathogen's presence to the host and trigger resistance. Indeed, snapshots of this evolutionary arms race in plant host-pathogen interactions have recently been presented (Allen et al. 2004, Wichmann et al. 2005). Additionally, there is increasing evidence that Avr gene products also have virulence functions (Gabriel 1999, van't Slot and Knogge 2002, White et al. 2000). Most evidence for this dual role for pathogen effector proteins comes from bacterial pathosystems (Anderson et al. 2006, Espinosa et al. 2003, Lim and Kunkel 2004, Losada et al. 2004, Nimchuk et al. 2001, Stevens et al. 1998, Tsiamis et al. 2000, Vivian and Gibbon 1997), although two fungal avirulence factors have been shown to function in virulence (Laugé and De Wit 1998, Laugé et al. 2000, Laugé et al. 1997, Laugé et al. 1998, Rohe et al. 1995, Wevelsiep et al. 1991). There is also evidence of Avr candidates with virulence functions in the oomycete pathogen Phytophthora infestans (Bos et al. 2003, Liu et al. 2005, Orsomando et al. 2001). Thus, it is conceivable that we have revealed a virulence factor with an avirulence function.

Interestingly, most fungal Avr genes thus far described (Catanzariti et al. 2006, Laugé and De Wit 1998, Rep et al. 2004, van't Slot and Knogge 2002), as well as some recently described oomycete Avr candidates (Bittner-Eddy et al. 2003, Bos et al. 2003), encode for small, secreted proteins with an even number of cysteine residues, characteristics shared with the products of PbToxB, ToxB, and toxb. Pb ToxB and Ptr toxb encode for 88 aa proteins, and Ptr ToxB encodes for an 87 aa protein, all of which have a 23 amino acid signal sequence and four shared cysteines (Chapter 3, Martinez et
al. 2001). Thus, it is conceivable that Pb ToxB is an avirulent remnant in the evolutionary history of Ptr ToxB. If we are indeed observing an arms race in action, that a Ptr ToxB homolog is found in *Magnaporthe grisea* (Chapter 3), causal agent of rice blast (Rossman et al. 1990b), suggests this “arms race” could have begun early in the history of the coevolution between ascomycetes and their grass hosts. Although determination of the role of Pb ToxB for *P. bromi* requires further investigation, a combination of *PbToxB* transcription and concomitant Pb ToxB activity on Ptr ToxB-sensitive wheat provides a compelling correlation for a function of Pb ToxB for *P. bromi* on wheat. Further investigation of the function of Pb ToxB promises to shed light on the evolutionary history of Ptr ToxB.
Chapter 5

Development of *ToxA* and *ToxB* promoter-driven fluorescent protein expression vectors for use in filamentous ascomycetes

Rachael M. Andrie, J. Patrick Martinez, and Lynda M. Ciuffetti

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810 E. 10th Street, Lawrence, KS 66044-8897
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Abstract

The green fluorescent protein (GFP) has been established as the premier in vivo reporter for investigations of gene expression, protein localization, and cell and organism dynamics. The fungal transformation vector pCT74, with sGFP under the control of the ToxA promoter from Pyrenophora tritici-repentis, effectively expresses GFP in a diverse group of filamentous ascomycetes. Due to the versatility of ToxA promoter-driven expression of GFP, we constructed an additional set of fluorescent protein expression vectors to expand the color palette of fluorescent markers for use in filamentous fungi. EYFP, ECFP, and mRFP1 were successfully expressed from the ToxA promoter in both its fungus of origin, P. tritici-repentis, and a distant relative, Verticillium dahliae. Additionally, the ToxB promoter from P. tritici-repentis drove expression of sGFP in V. dahliae, suggesting a similar potential to the ToxA promoter for heterologous expression in ascomycetes. The suite of fungal transformation vectors presented here promise to be useful for a variety of fungal research applications.

Introduction

Lorang and colleagues (2001) developed a versatile fungal transformation vector for the expression of the green fluorescent protein (GFP) in filamentous ascomycetes. Vector pCT74 employs the promoter from the Pyrenophora tritici-repentis necrosis-inducing host-selective toxin gene ToxA (Ciuffetti et al. 1997) to drive expression of the synthetic GFP gene, sGFP (Chiu et al. 1996). The form of sGFP used here possesses human-optimized codon usage, incorporates nucleotide changes that lead to a serine-to-threonine mutation at position 65 of the chromophore, and lacks a cryptic intron donor site that abolished GFP expression in Arabidopsis (Chiu et al. 1996). This vector was shown to confer bright cytoplasmic sGFP fluorescence to plant pathogens belonging to eight different genera of the Ascomycota where promoters from Aspergillus, Neurospora, and Colletotrichum had failed to produce acceptable levels of GFP fluorescence (Lorang et al. 2001). Although other fungal GFP expression vectors were also available, none had been evaluated in such a diverse range of fungi. Since that time, numerous members of
the Ascomycota have been successfully transformed with pCT74, including the model fungus *Neurospora crassa* (Freitag et al. 2001), as well as both *Trichoderma harzianum*, a known biocontrol agent, and *Ophiostoma picea*, a bluestain fungus of harvested lumber (Xiao et al. 2003). Thus, the ToxA promoter has proven very useful for strong expression of GFP in ascomycetes with diverse lifestyles.

Due to the success of ToxA promoter-driven sGFP expression in fungi of the Ascomycota, we sought to use the ToxA promoter to express additional fluorescent protein color variants. Since the isolation of the *gfp* gene from the jellyfish *Aequorea victoria* (Prasher et al. 1992) and demonstration of its utility as a fluorescent marker in heterologous organisms (Chalfie et al. 1994, Inouye and Tsuji 1994), there has been a continuous effort to develop new GFP variants with altered fluorescent properties (Lippincott-Schwartz and Patterson 2003, Tsien 1998). In addition to improving GFP fluorescence, mutagenesis studies have resulted in color variants with various absorbance and emission spectra. The development of the cyan fluorescent protein (CFP) (Heim and Tsien 1996) and yellow fluorescent protein (YFP) (Ormö et al. 1996) made simultaneous visualization of two distinct GFP variants practical (Ellenberg et al. 1998, Stuurman et al. 2000) and provided an ideal pair for fluorescent energy transfer (FRET) analysis (Dye et al. 2005, Tsien 1998). However, the production of a red mutant of GFP from *A. victoria* for use in multi-spectral imaging has met with little success (Czymmek et al. 2005, Lippincott-Schwartz and Patterson 2003).

Fortunately, red fluorescent proteins have been discovered in other marine organisms, although they fluoresce with variable efficacy (Fradkov et al. 2002, Gurskaya et al. 2001, Labas et al. 2002, Lukyanov et al. 2000, Matz et al. 1999, Palmer et al. 2004, Wiedenmann et al. 2002). The discovery of the first red fluorescent protein, DsRed, from a coral in the genus *Discoma* was well received (Matz et al. 1999), however its effectiveness was limited by slow maturation times, obligate oligomerization, and emission of fluorescence when excited at wavelengths optimal for GFP (Baird et al. 2000). Of the optimized forms of DsRed that have since been developed (Bevis and Glick 2002, Campbell et al. 2002, Terskikh et al. 2002, Verkhusha et al. 2001), mRFP1, a
monomeric form developed by Campbell et al (2002), shows promise as the superior partner with GFP for colocalization or FRET analysis and as the preferred candidate for construction of fusion proteins (Campbell et al. 2002, Zhang et al. 2002). In addition to the red variants, other reef coral fluorescent proteins (RCFPs) have also been added to the palette of available fluorescent proteins, including AmCyan, ZsGreen, and ZsYellow (Carter et al. 2004, Czymmek et al. 2005, Gurskaya et al. 2003, Labas et al. 2002, Matz et al. 1999). However, like DsRed, the other RCFPs are known to form oligomers and high molecular weight aggregates (Czymmek et al. 2005). Thus, from the GFP variants available, we chose EYFP, ECFP, and mRFP1 for inclusion in ToxA promoter-driven expression vectors. In combination with sGFP, these three colors provide a range of spectral profiles to address various challenges with autofluorescence (Tsien 1998), as well as two pairs of proteins with non-overlapping spectra ideal for in vivo colocalization studies and FRET analysis.

The nine copies of ToxB, a chlorosis-inducing host-selective toxin gene from P. tritici-repentis (Martinez et al. 2004, Martinez et al. 2001), potentially offer multiple promoters for heterologous expression of proteins in fungi. Although the putative promoters of five characterized ToxB loci share considerable sequence homology, some variation exists (Martinez et al. 2004). The presence or absence of certain sequence elements could contribute to differences in expression by each promoter (Gold et al. 2001). To initially assess heterologous expression from the putative ToxB promoter, we chose the ToxB1 promoter to express sGFP in a heterologous fungus, Verticillium dahliae.

We report here a new set of fluorescent protein expression vectors and an additional promoter for general use in filamentous ascomycetes. In addition to sGFP (Lorang et al. 2001), the ToxA promoter drives strong expression of EYFP, ECFP, and mRFP1 in both P. tritici-repentis and V. dahliae. In V. dahliae, the ToxB promoter drives comparable sGFP expression to that of the ToxA promoter, demonstrating its capacity for heterologous gene expression. We anticipate these vectors will be useful for the study of both fungal biology and fungi-host interactions.
Materials and methods

Fungal strains and culture conditions

This study used the *ToxA*-containing *P. tritici-repentis* isolate SD19, the *ToxB*-containing *P. tritici-repentis* isolate DW7 (Ali et al. 1999, Martinez et al. 2001), and a *V. dahliae* isolate from potato. *P. tritici-repentis* was grown on solid V8 agar (20% v/v V8, 0.3% w/v CaCO₃, 2% w/v Bacto agar) in constant darkness, 25 C. *V. dahliae* was grown on solid, half-strength potato dextrose agar (PDA; BD Difco, Sparks, MD) amended with 1 ppm streptomycin (FisherScientific, Pittsburgh, PA) in constant illumination at 25 C. *V. dahliae* sporulates under these conditions. However, *P. tritici-repentis* requires induction of conidiation by flooding a culture with sterile water and manually flattening the mycelia, followed by 18-24 h of light at room temperature and 18-24 h of dark at 16 C (Lamari and Bernier 1989a). Hygromycin-resistant transformants of *P. tritici-repentis* were transferred onto PDA with 15 µg/mL hygromycin B (Invitrogen, Carlsbad, CA); *V. dahliae* transformants were transferred onto half-strength PDA with 1 ppm streptomycin and 50 µg/mL hygromycin B.

Fluorescent protein expression vectors

Vector pCT73, which contains *sGFP* under the control of the *ToxA* promoter and *nos* terminator (Lorang et al. 2001), was digested with *NcoI* and *SmaI* to remove *sGFP-nos*. To make pCA42 and pCA43, *sGFP-nos* was replaced by *ECFP-nos* and *EYFP-nos* fragments from pAN94 and pAN95, respectively, which were generated via digestion with *SalI*, treatment to fill-in 5'-protruding ends, and digestion with *NcoI*. Both vectors were then digested with *SalI*, followed by ligation to an ~1.6 kb *SalI* hygromycin resistance cassette fragment (Carroll et al. 1994) from pCT48 (Ciuffetti et al. 1997) to produce pCA49 and pCA45. The orientation of the hygromycin B phosphotransferase gene, *hph*, was confirmed by sequence analysis (Central Services Lab, Center for Genome Research and Biocomputing, Oregon State University, Corvallis). The *hph* gene is in the opposite direction of *CFP* in pCA49 and *YFP* in pCA45. The final constructs are ~5.8 kb.
To produce pCA51, we used the following primers to engineer restriction enzymes sites on either side of mRFP1 (Campbell et al. 2002) from pCB302Hsp70hmRFP (Prokhnevsky et al. 2005) via polymerase chain reaction (PCR). The forward primer mRFP1 (5'-CATATCCCGGGATGGCCTCCTCCGAGGACGTCATC-3') introduced a 5' SmaI site, and the reverse primer mRFP2 (5'-CATATGGATCCAAGCTTTTAGGCGCCGGTGAGTG-3') introduced sites for HindIII and BamHI 3' of mRFP1. PCR was performed on ~10 ng plasmid DNA in a standard 50 µL reaction under standard PCR reaction conditions with a 62°C annealing temperature. The amplification product was ligated with pGEM-T Easy (Promega, Madison, WI) and a clone chosen by sequence analysis. Construct pCA51 was digested with SmaI and BamHI to release mRFP1, which was then inserted into pCT73 vector DNA that had been digested with NcoI, treated to fill-in 5'-protruding ends, digested with BamHI, and gel purified away from sGFP-nos. The resultant construct, pCA53, was digested with NotI for the insertion of an ~0.4 kb NotI nos-terminator fragment from pCT73 to form pCA54. The Sall hygromycin resistance cassette fragment was inserted into Sall-digested pCA54 to create pCA56. The resultant construct, pCA56, is ~5.9 kb.

For pCM56, a portion of the ToxB1 locus (GenBank accession AY425480) was amplified from genomic DNA of P. tritici-repentis isolate DW7 with primers TB21 and TB29 (Martinez et al. 2004). The PCR product was ligated with pGEM-T Easy to form pCM29. The putative ToxB promoter was PCR amplified from pCM29 with primers TB67 (5'-ATAATCGATTGTTGGAAGGCCTTGTAC-3') and TB68 (5'-GGCGCCATGGTCCTAACAAGGGAT-3'). The PCR product was digested with ClaI and NcoI and ligated with pCT73 that had been digested with ClaI and NcoI and purified to remove the ToxA promoter. The resulting plasmid, pCM50, was then digested with XhoI and ClaI and ligated to an XhoI/ClaI hygromycin resistance cassette fragment (Carroll et al 1994) to form pCM56.

All PCR products and gel fragments were purified with QIAquick Spin Kits (Qiagen, Valencia, CA). A QIAfilter Midi Plasmid Kit was used to purify high
concentrations of each construct. For transformation of *P. tritici-repentis*, constructs were linearized with *SacI* and concentrated via standard ethanol precipitation.

**Pyrenophora tritici-repentis** transformation

For *P. tritici-repentis*, transformation was via the protoplast method of Turgeon and colleagues (1985) with some modifications. Conidia were collected in water + 0.015% tween-20, inoculated into 100 mL quarter-strength potato dextrose broth (PDB; BD Difco, Sparks, MD), and grown at 25°C, 175 rpm, 16 h. After 16 h, fungal tissue was ground, inoculated into fresh media, and incubated for 2 h. Approximately 10 g of tissue was incubated in 100 mL enzyme-osmoticum (0.7 M NaCl, 3% β-D-glucanase [Interspex, San Mateo, CA] and 1% driselase [Sigma, St. Louis, MO]) for ~3 h at 30°C, 80 rpm. Protoplasts were collected through 100 μM pore-size nitex (Tetko Inc., Depew, NY). For each transformation reaction, 20 μg linear, plasmid DNA diluted 2-fold with STC (1.2 M sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl, pH 7.5) were added to 1 x 10⁷ total protoplasts in 200 μL. Transformation reactions were aliquoted equally between four plates of solid regeneration media (RM; 1.2 M sucrose, 0.1% yeast extract, 0.1% casein, 1.5% Bacto agar) at 2.5 x 10⁶ protoplasts per plate and spread with a glass rod. After ~24 to 36 h at 25°C, the plates were overlaid with 15 mL 1% water agar (pH 5.9) amended with 60 μg/mL hygromycin B for a final hygromycin B concentration of ~30 μg/mL. Hygromycin-resistant transformants were evident after two to three weeks.

**Verticillium dahliae** transformation

For *V. dahliae*, transformation was via the protoplast method of Dobinson (1995) with some modifications. Four flasks of 50 mL PDB + 0.001% thiamine were inoculated with *V. dahliae* spore solution to a final concentration of 6 x 10⁶ conidia/mL. PDB was prepared by boiling 200 g potatoes in 1 L e-pure water and filtering the suspension through cheesecloth. 20 g dextrose was added to the filtrate and the volume adjusted to 1 L with e-pure water. Stationary cultures of *V. dahliae* were aerated 8 h, followed by centrifugation at 1900 x g, 10 min, 4°C. Pellets were resuspended in 1 mL mycelia buffer...
(10 mM Na$_2$HPO$_4$, pH 7.5, 10 mM EDTA, pH 8.0) for a 30 min, room temperature incubation, followed by overnight digestion in enzyme-osmoticum (1 mg/mL Novozyme 234 in 1.2 M MgSO$_4$.7H$_2$O, 10 mM NaPO$_4$, pH 5.8) at 25 C, 65 rpm. Protoplasts were collected and transformed as described by Ciuffetti and colleagues (1997). For each transformation reaction, 15 μg circular plasmid DNA diluted two-fold with STC were added to a total of 1 × 10$^7$ protoplasts in 200 μL. Protoplasts were plated at 2 × 10$^6$ protoplasts per plate. Hygromycin-resistant colonies were evident after one week.

**Fungal slide mounts**

Three slide mounting techniques were used: (i) a square of media was placed on a slide, squashed with a cover slip, and sealed with clear nail polish; (ii) 20 μL of a conidial suspension were added to a slide, cover-slipped, and sealed; and (iii) the touch tape method developed by Harris (2000) was modified as follows. A wood applicator was attached to one end of a piece of clear adhesive tape. The free end of the tape was horizontally cut to allow for ease of detachment from the applicator. The tape was touched to a fungal mat and inverted on a microscope slide sticky side up. A cover slip was then placed over the sample and sealed for microscopic analysis. The media mount was used for both species for both confocal and fluorescence microscopy. The conidia mount was used in confocal microscopy of *P. triticum-repentis* and the tape mount was used in fluorescence microscopy of *V. dahliae*.

**Microscopy**

A Leica DMRB epifluorescence microscope (Leica Microsystems, Wetzler, Germany) or a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany) was used to observe slides of fluorescent transformants. The epifluorescence microscope was fitted with a mercury lamp and the appropriate filter sets (Chroma Technology Corp., Rockingham, VT) for visualization of sGFP (Endow GFP Bandpass Emission Set: HQ470/40x exciter, Q495LP dichroic, HQ525/50m emitter), EYFP (Yellow GFP BP [10C/Topaz] Set: HQ500/20x, exciter, Q515LP dichroic,
HQ535/30m emitter), and mRFP1 (TRITC [Rhodamine]/Dil Red Shifted Emission Set: HQ535/50x exciter, Q565LP dichroic, HQ620/60m emitter). A Leica MZFLIII stereomicroscope equipped with a mercury lamp and the GFP Plant fluorescence filter set (470/40 nm excitation filter, 525/50 nm barrier filter; Chroma) was used to image the hyphae of *V. dahliae* transformed with *ToxB* promoter-driven sGFP. Image-Pro PLUS in conjunction with a CoolSNAP-Pro digital camera (MediaCybernetics, Silver Spring, MD) was used to capture images from both the epifluorescence microscope and stereomicroscope.

For confocal microscopy, sGFP, EYFP, and ECFP were excited with an argon laser at 10%, 10%, and 17%, respectively, whereas a HeNe laser was used at 80% for mRFP1. The following beam splitter and filter configurations were used: (i) sGFP: HFT488, LP505; (ii) EYFP: HFT405/514, NFT490, BP530-600; (iii) mRFP1: HFT488/543, NFT545, LP560; and (iv) ECFP: HFT458, NFT515, BP470-500. The pinhole values for sGFP, EYFP, mRFP1, and ECFP were 114 μm, 114 μm, 127 μm, and 431 μm, respectively. Images were reconstructed in Zeiss LSM 510 software.

For both fluorescence and confocal microscopy, slides of wildtype fungi were viewed under all settings as a check for background autofluorescence (data not shown).

**Results**

Vector pCT74 (Carroll et al. 1994, Lorang et al. 2001), which relies on antibiotic resistance conferred by a modified form of the *E. coli* hygromycin B phosphotransferase (*hph*) gene (Carroll et al. 1994, Lorang et al. 2001) for selection, was used as a template for the construction of transformation vectors with the *ToxA* promoter driving expression of an additional set of fluorescent proteins. Genes for EYFP, ECFP, and mRFP1 were cloned downstream of the *ToxA* promoter to produce the constructs pCA45, pCA49, and pCA56, respectively. Sequence analysis confirmed successful vector construction.

To test the ability of the *ToxA* promoter (Ciuffetti et al. 1997) to drive expression of these fluorescent proteins, the resultant constructs were transformed into two fungal species known to successfully express sGFP from the *ToxA* promoter (Ciuffetti et al.
Positive transformants of *P. tritici-repentis*, the source of the *ToxA* promoter, and *V. dahliae*, an ascomycete distantly related to *P. tritici-repentis* (Lutzoni et al. 2004), were first chosen for their ability to grow under hygromycin selection, followed by confirmation of EYFP and mRFP1 fluorescence via fluorescence or confocal microscopy, and ECFP fluorescence via confocal microscopy. For hygromycin-resistant transformants of both *P. tritici-repentis* and *V. dahliae*, wildtype colony morphology and conidiation were confirmed (data not shown). Confirmation of fluorescence by each type of transformant revealed a range of intensities, although only a few lacked any fluorescence (data not shown). The brightest individual of each was chosen for subsequent analysis.

As shown by fluorescence and confocal microscopy, the *ToxA* promoter successfully drove expression of all three fluorescent proteins, EYFP, ECFP, and mRFP1, in the cytoplasm of both *P. tritici-repentis* and *V. dahliae* (Figure 5.1). Confocal imaging revealed bright EYFP (Figure 5.1B) and mRFP1 (Figure 5.1D) fluorescence in conidia and conidiophores of *P. tritici-repentis*, commensurate with the sGFP fluorescence of the original pCT74 transformant (Figure 5.1A) (Sawyer et al. 1998). ECFP fluorescence also occurred in conidia (Figure 5.1C) and conidiophores (data not shown), but required maximized settings to obtain a signal as intense as the other three fluorescent proteins (Figure 5.1A, B, and D). EYFP, ECFP, and mRFP1 fluorescence also was present in *P. tritici-repentis* hyphae (data not shown). *V. dahliae* revealed a similar pattern of fluorescence as *P. tritici-repentis* (Figure 5.1E-H). Fluorescence microscopy showed that EYFP and mRFP1 expression in conidia, conidiophores, and hyphae of *V. dahliae* is similar to *ToxA* promoter-driven sGFP expression (Sawyer et al. 1998), whereas confocal imaging of the same set of structures again required maximized settings to acquire a readily detectable ECFP signal (Figure 5.1E-H). Additionally, *V. dahliae* sclerotia possess sGFP (Sawyer et al. 1998), EYFP, and mRFP1 fluorescence to
Figure 5.1. *Pyrenophora tritici-repentis* and *Verticillium dahliae* express a suite of fluorescent proteins from the *P. tritici-repentis ToxA* promoter.

A-D) Confocal micrographs of *P. tritici-repentis* conidia transformed with sGFP (A), EYFP (B), ECFP (C), and mRFP1 (D). Scale bar = 10 μm. E, F and H) Fluorescence micrographs of *V. dahliae* transformed with sGFP (E), EYFP (F), and mRFP1 (H). Fluorescence micrographs represent colors observed with the filter sets used in this study. As such, EYFP (F) appears more green than yellow. Scale bar = 5 μM. G) Confocal micrograph of *V. dahliae* transformed with ECFP. Scale bar = 5 μm.
Figure 5.1. *Pyrenophora tritici-repentis* and *Verticillium dahliae* express a suite of fluorescent proteins from the *P. tritici-repentis* ToxA promoter.
varying degrees (data not shown). However, ECFP fluorescence was not detectable (data not shown), possibly because it was masked by melanization of sclerotia cell walls. Finally, in contrast to *P. tritici-repentis*, which had a near uniform distribution of fluorescence among conidia (Figure 5.1A-D), *V. dahliae* conidia showed a heterogeneous range of intensities for all four proteins (Figure 5.1E-H).

To assess the utility of the ToxB promoter for heterologous expression, a portion of the ToxB1 promoter was fused to sGFP to create the transformation vector pCM56 (Figure 5.2A). Transformation of *V. dahliae* with pCM56 produced hygromycin-resistant colonies that grew and sporulated similarly to wildtype. Fluorescence microscopy revealed that, like the ToxA promoter, the ToxB promoter drives strong expression of sGFP in conidia, conidiophores, and hyphae of *V. dahliae* (Figure 5.2B and C). Sclerotia also displayed ToxB promoter-driven sGFP fluorescence (data not shown). Additionally, pCM56-transformed *V. dahliae* spores exhibited an array of sGFP intensities similar to that observed for ToxA promoter-driven sGFP expression (Figure 5.2C).

**Discussion**

This study expands the repertoire of fluorescent transformation vectors available for use in filamentous ascomycetes (Lorang et al. 2001, Mikkelsen et al. 2003, Pöggeler et al. 2003, Toews et al. 2004). As recently reviewed by Czymmek and colleagues (2005), live imaging using fluorescent proteins has advanced the study of fungal cell biology, phytopathology, and fungal ecology via a number of applications, including as reporters of gene expression or protein distribution, as tags of subcellular compartments or whole fungi, and as tools to study protein kinetics or to measure protein-protein interactions via FRET. To provide an arsenal of colors useful for a variety of applications, we chose the fluorescent proteins EYFP, ECFP and mRFP1 to join sGFP for inclusion in ToxA promoter-driven fluorescent vectors for fungal transformation. Successful expression of these three additional fluorescent proteins by the ToxA promoter in the cytoplasm of both its fungus of origin, *P. tritici-repentis* (Figure 5.1A-D), and a
Figure 5.2. The ToxB promoter from *Pyrenophora tritici-repentis* expresses sGFP fluorescence in *Verticillium dahliae*.

A) Map of the transformation vector pCM56, which harbors *sGFP* under the control of the ToxB promoter and modified *E. coli* *hph* under the control of the trpC promoter from *Aspergillus nidulans*. B) Fluorescence micrograph of ToxB promoter-driven sGFP expression in the hyphae of *V. dahliae*. Scale bar = 50 μm. C) Fluorescence micrograph of a *V. dahliae* fruiting body expressing sGFP from the ToxB promoter. Scale bar = 5 μm.
Figure 5.2. The ToxB promoter from *Pyrenophora tritici-repentis* expresses sGFP fluorescence in *Verticillium dahliae.*
distantly-related ascomycete, *V. dahliae* (Figure 5.1E-H), suggests these constructs will have as wide ranging applications as the original *ToxA* promoter-driven sGFP construct, pCT74 (Lorang et al. 2001). In addition, a new sGFP expression vector, pCM56, with *sGFP* under the control of the *ToxB* promoter (Figure 5.2A), was assembled. Strong sGFP expression in *V. dahliae* by the *ToxB* promoter (Figure 5.2B and C) revealed the potential of this promoter for heterologous expression of proteins in filamentous ascomycetes.

Confocal parameters used in this study revealed that fluorescence due to EYFP and mRFP1 is of similar intensity to that of sGFP, whereas ECFP fluorescence is considerably less intense. The maximized settings required to obtain intensities of ECFP similar to the other fluorescent proteins may be explained by its intrinsic brightness, which is the product of the extinction coefficient, a measure of light absorption ability, and quantum yield, the amount of absorbed light energy that is released as fluorescence (Tsien 1998). Based on this calculation, the intensity of ECFP is expected to be approximately 40% that of EGFP (Cubitt et al. 1999, Patterson et al. 2001, Tsien 1998), congruent with our observations. In contrast, mRFP1 did not require maximized confocal parameters even though its intrinsic brightness is calculated to be 30% of EGFP (Campbell et al. 2002), a value comparable to that for ECFP. A microenvironment favorable to mRFP1 fluorescence, but not to ECFP fluorescence, may explain why mRFP1 appeared more intense than ECFP despite similar calculated values of intrinsic brightness. Indeed, multiple environmental factors, such as temperature and pH, are known to differentially influence brightness over the range of available fluorophores (Cubitt et al. 1999, Patterson et al. 1997, Tsien 1998). An alternate explanation might be that the extinction coefficient and quantum yield contribute unequally to the overall brightness of a particular fluorophore. The fluorescence of mRFP1, which relies on the strength of its extinction coefficient as the principal contributor to brightness, is more intense than that of ECFP, which attributes its intensity primarily due to its quantum yield (Cubitt et al. 1999, Patterson et al. 2001, Tsien 1998). When comparing these two fluorescent proteins, it appears that the extinction coefficient rather than the quantum
yield contributes more to overall brightness. Similarly, Patterson and colleagues (1997) found that mutant forms of GFP with increased extinction coefficients are approximately six-fold brighter than wildtype GFP in spite of corresponding decreases in quantum yields. Cerulean (Rizzo et al. 2004), an improved CFP variant, could provide a brighter alternative to ECFP.

From the fungi successfully transformed with ToxA-driven GFP fluorescence (Lorang et al. 2001), we chose V. dahliae for transformation with the ToxB promoter-driven sGFP construct, pCM56, due to its taxonomic and pathogenic divergence from P. tritici-repentis. Although V. dahliae and P. tritici-repentis are in the same subphylum, the Pezizomycotina, they diverge at the level of class and thus show considerable phylogenetic distance (Lutzoni et al. 2004). Additionally, V. dahliae primarily infects dicotyledenous plant hosts (Pegg and Brady 2002), whereas P. tritici-repentis infects monocotyledonous grass species (Strelkov and Lamari 2003). Thus, we reasoned that if the ToxB promoter expresses GFP in V. dahliae, we might expect the ToxB promoter to have as wide ranging applicability for heterologous expression as the ToxA promoter. That the ToxB promoter rivals the ToxA promoter in driving stable, constitutive GFP expression in V. dahliae (Figures 5.1E, 5.2B and C) gives us confidence that the ToxB promoter will be a useful tool for heterologous expression, at least across the Ascomycota. Furthermore, it will be advantageous to eventually analyze expression from each distinct promoter configuration of the multi-copy ToxB gene. In light of variable levels of expression in fungi from heterologous promoters (Churchill et al. 1990, Gold et al. 2001, Lorang et al. 2001, Spellig et al. 1996, Van Wert and Yoder 1994), and that fungal gene expression systems often simultaneously use multiple promoters, one fused to a gene of interest and another fused to a selectable marker (Gold et al. 2001, Lorang et al. 2001), the advent of new fungal promoters with the potential for widespread application should prove very advantageous.

A number of other new constructs for transformation of filamentous fungi are available that utilize various combinations of promoters and fluorescent protein variants. The greatest diversity of new fungal fluorescent marker constructs has been provided by
in planta investigations of plant pathogens. For example, the *A. nidulans gpd* promoter successfully expressed DsRed2 (Nahalkova and Fatehi 2003) and sGFP (Aboul-Soud et al. 2004) in *Fusarium oxysporum f. sp. lycopersici*; the homologous ribosomal protein 27 promoter expressed four GFP variants in a barley-infecting isolate of *Magnaporthe grisea* (Czymmek et al. 2002); and various promoters were used to express a suite of RCFPs in the plant pathogens *M. grisea* and *Fusarium verticillioides* (Bourett et al. 2002), as well as *Colletotrichum graminicola* and *F. oxysporum* (Czymmek et al. 2005).

In addition to those generated for in planta observations of fungi, fluorescent expression vectors have been developed to answer basic questions about fungal cell biology. The cytoplasmic ZsGreen constructs developed by Bourett and colleagues (2002) were used to monitor subcellular organelle dynamics and changes in nuclear envelope permeability in *M. grisea* and *F. verticillioides* via fluorescent protein exclusion from organelles. Czymmek and colleagues (2005) found the use of the RCFPs ZsGreen and AmCyan for localization to organelles to be fraught with difficulties, however they successfully localized EGFP and AcGFP, a GFP variant from *A. coerulescens* (Gurskaya et al. 2003), to mitochondria of *M. grisea*. They also used non-native promoters to express β-tubulin-EYFP fusion proteins for the visualization of microtubules during mitosis. In contrast, to study mitosis in the model organisms *N. crassa* and *A. nidulans*, homologous promoters were used to drive expression of fluorescent protein fusions to tubulin or histone proteins for the analysis of in situ microtubule or nuclear dynamics, respectively. For *N. crassa*, both histone H1 and β-tubulin were fused to sGFP and analyzed in separate individuals (Freitag et al. 2004); whereas, for *A. nidulans*, histone H2A was fused to CFP and α-tubulin was fused to GFP for simultaneous imaging in one individual (Su et al. 2004). Finally, to study protein-protein interactions of transcription factors in *Acremonium chrysogenum*, Hoff and Kück (2005) used EYFP for biomolecular fluorescence complementation. The heterologous glyceraldehyde-3-phosphate (*gpd*) promoter from *A. nidulans* was used to drive expression of the N- and C-termini of EYFP fused to two different transcription factors suspected to interact. Interaction of the two
transcription factors in the nucleus brought the two domains of EYFP into proximity of each other and restored EYFP fluorescence.

The tremendous success of the GFP as an in vivo reporter has revolutionized our understanding of fungal biology and fungi-host interactions (Czymmek et al. 2005, Jensen and Schulz 2004, Lorang et al. 2001). Advances in fluorescent protein technology allowed for the expansion of the color palette of fluorescent transformation vectors available for use in filamentous fungi. This study describes a new set of fluorescent protein expression vectors that we anticipate will be useful for a variety of applications in fungal-related research areas. Although the vectors presented here result in the labeling of whole fungi, we predict that the ToxA and ToxB promoters can also be used to drive expression of fluorescent protein fusions for the determination of protein distribution or the labeling of specific cell components or organelles. Additionally, these promoters show promise for heterologous expression in ascomycetes of any protein of choice.
Chapter 6

General Conclusions

*Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, is an economically important pathogen of wheat worldwide. It produces multiple host-selective toxins (HSTs), including *Ptr ToxA*, *Ptr ToxB*, and *Ptr ToxC*. These three HSTs are variably distributed among different isolates of *P. tritici-repentis*, both singularly and in all possible combinations, resulting in a complex race structure for this fungus. *P. tritici-repentis* race identification is typically based on inoculation of a standard set of host differentials, similar to the race designation system for cereal rusts. However, we also found that a combination of phenotypic and genotypic characterization can be used to designate new races of *P. tritici-repentis*, and in some cases, is necessary to reveal variability in *P. tritici-repentis* not apparent by phenotype alone. In particular, inoculation of two *P. tritici-repentis* isolates on the standard wheat differential set indicated their classification as known races of *P. tritici-repentis*. However, genetic evaluation revealed they do not have all of the HST genes, and therefore do not produce all of the toxins, expected for phenotypically-derived race assignments. In the process, we identified two new races of *P. tritici-repentis* that apparently produce phytotoxic activities distinct from *Ptr ToxA*, *Ptr ToxB*, and *Ptr ToxC*. Based on these results, I conclude that both phenotype and genotype should be used for race classification.

To aid in race classification, we took advantage of the intimate association between HST production and race identity to develop a straight-forward and high-throughput multiplex PCR assay based on HST gene-specific primers. The ease with which PCR can be executed will facilitate rapid characterization of *P. tritici-repentis* isolates, as well as amenable to the inclusion of additional primer pairs for the detection of additional disease determinants as they are discovered and their genetic determinants are characterized. Although genotypic race characterization alone cannot yet replace phenotypic race designation, foremost because all of the disease determinants
involved in the interaction between *P. tritici-repentis* and wheat have yet to be characterized, its application promises to help minimize laborious inoculation screens involved in race characterization, both in individual laboratories and as a part of breeding efforts for tan spot resistance in wheat.

The application of our multiplex PCR assay to various isolates of *P. tritici-repentis* and its relatives led to the discovery that ToxB-like sequences are present in *P. bromi*, the species most closely related to *P. tritici-repentis*. Sequence comparison of the multiple ToxB-like sequences in *P. bromi*, since designated *PbToxB*, to ToxB from *P. tritici-repentis* revealed the obvious homology of these sequences. Superimposition of the results of Southern analysis on a phylogram of *P. tritici-repentis* and its Pleosporalean relatives suggested that the distribution of ToxB extends beyond the races of *P. tritici-repentis* to other grass pathogens of the Pyrenophora and Pleosporaceae. Although I only sampled representative members of each clade, it is conceivable that other relatives in the hierarchy also possess ToxB-like sequences. Additionally, a search of available fungal genomes identified a distant homolog in *Magnaporthe grisea*, causal agent of rice blast and a member of a different class than that to which *P. tritici-repentis* belongs. The presence of putative ToxB-like sequences across a broad range of plant pathogenic ascomycetes provides further support for the hypothesis that *P. tritici-repentis* coevolved with various grass hosts prior to moving to its agricultural host, wheat. Additionally, the continued maintenance of ToxB-like sequences in a range of fungal lineages raises the question as to what selective advantage homlogs of ToxB confer to organisms in which they exist.

Due to the relatedness of *P. tritici-repentis* and *P. bromi*, and that of their grass hosts, it is conceivable that *P. bromi* evolved similar disease mechanisms as *P. tritici-repentis*. Thus as a first step in the functional characterization of the homologs of ToxB, I investigated the possibility that *PbToxB* encodes for a HST in brownspot of bromegrass caused by *P. bromi*. Due to a lack of activity of heterologously-expressed Pb ToxB proteins on bromegrass, I concluded that Pb ToxB does not act as an HST for *P. bromi*, at least on tested bromegrass. However, *PbToxB* is actively transcribed, thus presumably
plays a role in *P. bromi* biology. Thus, I subsequently examined whether any of the various Pb ToxB proteins had activity on wheat. Inoculation of *P. bromi* elicits a resistance response on Ptr ToxB-sensitive wheat, but subsequent infiltration of various Pb ToxB proteins resulted in a range of activities, from no activity to activity similar to that of Ptr ToxB itself. Activity differences presumably reflect the one to two amino acid differences present in the various Pb ToxB proteins.

Demonstration that the PbToxB transcript is expressed in *P. bromi*-inoculated wheat as determined by *in planta* reverse-transcription polymerase chain reaction suggested that PbToxB may serve a functional role for *P. bromi* on tested wheat differentials. It is possible that Pb ToxB has the potential to establish a disease interaction between *P. bromi* and wheat similar to that of Ptr ToxB, however *P. bromi* possess too few copies of PbToxB to overcome disease resistance responses. Indeed, evidence suggests that *P. tritici-repentis* requires either the presence of multiple functional copies of ToxB or high levels of Ptr ToxB production to be pathogenic on Ptr ToxB-sensitive wheat. The *P. bromi* isolates tested only have two copies of PbToxB. Alternatively, Pb ToxB may mediate a resistance response between *P. bromi* and wheat, thus function as an avirulence factor. If so, homologs of ToxB may represent evolutionary remnants of an arms race between ascomycetes and their grass hosts. Functional characterization of ToxB homologs for the fungi in which they are found, in addition to a more robust sampling, promises to illuminate the evolutionary history of ToxB.

Several valuable tools were developed as a result of this work, both for the investigation of the interaction between *P. tritici-repentis* and wheat, and for the investigation of host-pathogen interactions and fungal biology in general. First, as already mentioned, the ease with which our multiplex PCR can be executed should provide a useful complement to disease phenotype in the classification of *P. tritici-repentis* isolates to race. Second, the multigene phylogeny used to investigate the distribution of ToxB will provide a phylogenetic framework within which to investigate various evolutionary hypotheses. The most obvious immediate application is to direct
cloning efforts of ToxB homologs to facilitate their functional characterization. Additionally, in an effort to investigate the evolutionary history of this HST, identified ToxB homologs can also be used to construct a ToxB gene genealogy for comparison to the species phylogeny. The phylogeny could also be applied to the investigation of evolutionary hypotheses regarding ToxA. In particular, the ability to transform a nonpathogenic isolate into a pathogen with just one gene, ToxA, despite the genetic diversity present between isolates of P. tritici-repentis, suggests that pathogenic potential may be more important than strict genetic relatedness with regard to the ability to become a pathogen on wheat upon acquisition of Ptr ToxA production. To investigate the relative importance of pathogenic potential and genetic relatedness, our phylogeny can be used to direct hierarchical transformation studies with the ToxA gene.

Finally, we constructed a new set of fluorescent protein expression vectors that we anticipate will be useful for a variety of applications in fungal-related research areas. In addition to sGFP, the ToxA promoter drove strong expression of EYFP, ECFP, and mRFP1 in both P. tritici-repentis and Verticillium dahliae, suggesting these constructs will have as wide-ranging applications as the original ToxA promoter-driven sGFP construct, pCT74. Additionally, the ToxB promoter drove comparable sGFP expression to that of the ToxA promoter in V. dahliae, demonstrating its capacity for heterologous gene expression. In light of variable levels of expression in fungi from heterologous promoters and that fungal gene expression systems often simultaneously use multiple promoters, one fused to a gene of interest and another fused to a selectable marker, the advent of new fungal promoters with the potential for widespread application should prove very advantageous. Although the vectors presented here result in the labeling of whole fungi, we predict that the ToxA and ToxB promoters can also be used to drive expression of fluorescent protein fusions for the determination of protein distribution or the labeling of specific cell components or organelles. Additionally, these promoters show promise for heterologous expression in ascomycetes of any protein of choice. Thus, we anticipate these vectors will be useful for the study of both fungal biology and fungi-host interactions.
Bibliography


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