***Pyrenophora bromi*, Causal Agent of Brownspot of Bromegrass, Expresses a Gene Encoding a Protein with Homology and Similar Activity to Ptr ToxB, a Host-Selective Toxin of Wheat**

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Submitted 26 June 2010. Accepted 10 November 2010.

Ptr ToxB, encoded by *ToxB*, is one of multiple host-selective toxins (HST) produced by the wheat pathogen *Pyrenophora tritici-repentis*. Homologs of *ToxB* are found in several ascomycetes, including sister species *Pyrenophora bromi*, causal agent of brownspot of bromegrass. Due to the close evolutionary relatedness of *P. tritici-repentis* and *P. bromi* and that of their grass hosts, we hypothesized that homologs of *ToxB* in *P. bromi* may act as HST in the disease interaction between *P. bromi* and bromegrass. A representative set of transcriptionally active *P. bromi* *ToxB* genes were heterologously expressed in *Pichia pastoris* and the resultant proteins tested for their ability to act as HST on bromegrass. The tested *Pyrenophora bromi* *ToxB* (Pb ToxB) proteins were not toxic to bromegrass; thus, Pb ToxB does not appear to function as an HST in the *P. bromi*–bromegrass interaction. Instead, we revealed that the Pb ToxB proteins can be toxic to Ptr ToxB-sensitive wheat, at levels similar to Ptr ToxB, and the corresponding *P. bromi* *ToxB* genes are expressed in *P. bromi*-inoculated wheat. Our data suggest that *P. bromi* possesses the potential to become a wheat pathogen and highlights the importance of investigating the interaction between *P. bromi* and wheat.

Ptr ToxB (Orolaza et al. 1995; Strelkov et al. 1999) is one of multiple host-selective toxins (HST) 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). It is a small, 6.5-kDa protein encoded by the *ToxB* gene (Martinez et al. 2001) that induces chlorosis on the sensitive wheat differentials Katepwa and 6B662 (Lamari et al. 1995; Orolaza et al. 1995). Isolates of *P. tritici-repentis* are separated into races based on the complement of HST that they produce, with races 5 (Lamari et al. 1995), 6 (Strelkov et al. 2002), 7, and 8 (Lamari et al. 2003) containing up to an estimated 10 copies of Ptr ToxB-encoding genes, depending on the isolate (Lamari et al. 2003; Martinez et al. 2004; Strelkov et al. 2006). Furthermore, although they do not produce Ptr ToxB and are not pathogenic on Ptr ToxB-sensitive wheat, *P. tritici-repentis* races 3 and 4 each contain single-copy homologs of *ToxB*. In race 3, all but the first six nucleotides of the *ToxB*-like sequence are identical

to *ToxB* (Strelkov and Lamari 2003; Strelkov et al. 2006); whereas, for race 4, the sequences are more divergent (Martinez et al. 2004; Strelkov et al. 2006). The remaining non-Ptr ToxB-producing races of *P. tritici-repentis* lack *ToxB* homologs (Andrie et al. 2007; Martinez et al. 2004) but require other HST to be pathogenic (Andrie et al. 2007; Balance et al. 1996; Ciuffetti and Tuori 1999; De Wolf et al. 1998; Strelkov and Lamari 2003).

All of the *ToxB* homologs in *P. tritici-repentis* have been shown to be transcriptionally active. Initially, it was thought that transcription of the *ToxB* homologs in race 3, race 4, and a weakly virulent race 5 isolates was restricted to conidia (Strelkov et al. 2006), which is in contrast to both mycelial and conidial transcription of *ToxB* in a highly virulent race 5 isolate (Martinez et al. 2004; Strelkov et al. 2006). However, more sensitive detection methods have since shown that all *P. tritici-repentis* *ToxB* homologs are expressed in both mycelia and conidia (Amaike et al. 2008). In addition, *ToxB* appears to confer virulence in a copy-number-dependent manner: i) a virulent race 5 isolate has a higher copy number and levels of *ToxB* expression than a weakly virulent race 5 isolate with fewer copies and lower transcript levels (Amaike et al. 2008) and ii) *ToxB*-containing transformants with a higher copy number are more virulent when inoculated on Ptr ToxB-sensitive wheat than transformants with fewer copies (Ciuffetti et al. 2010).

Although evidence suggests that race 4 isolates of *P. tritici-repentis* that contain a *ToxB* homolog (for convenience designated *tox*b and the corresponding protein designated Ptr *tox*b) possess a general pathogenic ability (Ciuffetti et al. 1997, 2010; Lamari and Bernier 1989; Larez et al. 1986), thus far they have not been shown to cause disease (Amaike et al. 2008; Cao et al. 2009). The absence of chlorosis-inducing activity by these isolates appears to be due to a combination of the extent of *tox*b/Ptr *tox*b expression and sequence differences in the gene or protein (Kim and Strelkov 2007). Not only is Ptr *tox*b undetectable in race 4 mycelium, spore germination fluids, and culture filtrates (Cao et al. 2009) but a combination of sequence and phylogenetic analyses suggests that *tox*b may be evolving toward a fate as a pseudogene (Andrie et al. 2008).

Recently, *ToxB* homologs also have been discovered in a broad range of plant-pathogenic ascomycetes (Andrie et al. 2008), including *Pyrenophora bromi*, the sister species of *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). Due to the relatedness of *P. bromi* and *P. tritici-repentis* and

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their grass hosts, we hypothesized that the *P. bromi* *ToxB* homologs encode for HST involved in the interaction between *P. bromi* and bromegrass. In fact, due to the presence of chlorotic halos beyond the boundaries of fungal hyphae, a phyto-toxin has been implicated in brownspot of bromegrass caused by *P. bromi* (Sherwood 1996). Furthermore, the *P. bromi* *ToxB* sequences appear to be conserved (Andrie et al. 2008).

To test the hypothesis that various *P. bromi* *ToxB* homologs encode for HST, we employed the approach developed by Martinez and associates (2001) for the characterization of the role of *ToxB* in the interaction between *P. tritici-repentis* and wheat. We heterologously expressed various transcriptionally active *P. bromi* *ToxB* sequences in a yeast expression system, followed by assessment of their toxicity on bromegrass. Although our results show that the products of the *P. bromi* *ToxB* genes do not function as HST in the interaction between *P. bromi* and bromegrass, we reveal that they are toxic to wheat, suggesting a potential for *P. bromi* to become a wheat pathogen.

RESULTS

The *ToxB* homologs in *P. bromi* are transcriptionally active.

To confirm that the *ToxB* homologs in *P. bromi* (for convenience tentatively named *Pb ToxB*, and the corresponding proteins named *Pb ToxB*, to differentiate their species origin as *P. bromi*) (Andrie et al. 2008) are actively transcribed, we used semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to analyze RNA from *P. bromi* grown under conditions in which pathogenic *P. tritici-repentis* race 5 isolate DW7 is known to produce *ToxB* mRNA (Martinez et al. 2004). We first confirmed that our *ToxB* primers, TB7 and TB59, efficiently amplify both *Pb ToxB* and *ToxB* from *P. bromi* and *P. tritici-repentis* genomic DNA, respectively (data not shown). The results of semiquantitative RT-PCR analysis with these primers showed that various *Pb ToxB* genes from *P. bromi* are actively transcribed in culture (Fig. 1A). Furthermore, a comparison of the RT-PCR results for *P. bromi* isolates SM101 and SM106 (Fig. 1A) with the RT-PCR results for *P. tritici-repentis* isolate DW7 (Fig. 1B) suggests that the level of *Pb ToxB* expression is less than that for *ToxB* under the same conditions. Because we normalized the amount of fungal biomass in each PCR reaction by using the same amount of starting RNA for first-strand cDNA synthesis, adding the same amount of cDNA in subsequent PCR reactions, and loading the same amount of

Fig. 1. *Pyrenophora bromi* *ToxB* (*Pb ToxB*) homologs are actively transcribed in culture, although at lower levels than *ToxB* from a pathogenic *P. tritici-repentis* race 5 isolate. **A**, Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of *Pb ToxB* expression from *P. bromi* isolates SM101 and SM106. **B**, RT-PCR analysis of *ToxB* expression from *P. tritici-repentis* race 5 isolate DW7. Amplification intensities were comparable between all isolates because equal amounts of starting RNA and cDNA were used for first-strand cDNA synthesis and subsequent PCR reactions, respectively. Equal amounts (15 μ l) of MassRuler DNA Ladder, Mix, ready-to-use (MBI Fermentas, Hanover, MD, U.S.A.) were loaded on each agarose gel. Band sizes are given in base pairs. Mkr = DNA mass ladder, *Pb* = *P. bromi*, *Ptr* = *P. tritici-repentis*.

DNA ladder on each gel, the intensity differences observed in Figure 1 should reflect quantitative differences between *ToxB* and *Pb ToxB* transcript levels. Similar results were obtained for *P. bromi* isolate Bf-1 (data not shown).

Heterologous expression of representative *Pb ToxB* homologs.

To determine whether various *Pb ToxB* homologs function as HST in the *P. bromi*–bromegrass interaction, we heterologously expressed representative *Pb ToxB* homologs following the approach developed by Martinez and associates (2001) used to confirm that *ToxB* encodes for the HST Ptr ToxB. *P. tritici-repentis* *ToxB* and *tox* were similarly expressed to be used as positive and negative controls, respectively, of Ptr ToxB-induced symptom development. Representative *Pb ToxB* homologs were chosen for heterologous expression in *Pichia pastoris* based on the phylogenetic relationships of the deduced *Pb ToxB* amino acid sequences (Fig. 2A). At least one

Fig. 2. Heterologous expression of *ToxB* homologs from *Pyrenophora bromi*. **A**, A subset of *Pb ToxB* genes was chosen for heterologous expression based on their phylogenetic relationships as revealed by a distance analysis of the deduced *Pb ToxB* amino acid sequences (Andrie et al. 2008). Chosen loci are demarcated with a box. **B**, Culture filtrates containing heterologously expressed *Pb ToxB*, *Ptr tox*, and *Ptr ToxB* proteins were analyzed by 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Each culture filtrate was loaded in duplicate and paired lanes are grouped by black lines above protein names. The gel was silver-stained for densitometric quantitation of the expressed proteins (average of duplicate band intensities) via comparison with a set of known amounts of homogeneous native *Ptr ToxB* standards (Std), including 75, 150, 225, and 350 ng (from left to right).

representative from each of the three observed *Pb ToxB* clades was chosen for heterologous expression. Furthermore, two additional *Pb ToxB* genes were also chosen for expression, such that all of the protein products found in the *P. bromi* isolates SM101, SM106, and Bf-1 were represented. In total, five *Pb ToxB* loci were chosen for heterologous expression, including *Pb*(SM106) *ToxB1* and *Pb*(SM106) *ToxB2* from *P. bromi* isolate SM106, *Pb*(SM101) *ToxB1* and *Pb*(SM101) *ToxB2* from *P. bromi* isolate SM101, and *Pb*(Bf-1) *ToxB1* from *P. bromi* isolate Bf-1. Furthermore, as shown in the tree in Figure 2A, *Pb*(Bf-1) *ToxB2* and *Pb*(TAM115) *ToxB1* are identical to *Pb*(Bf-1) *ToxB1*, and *Pb*(TW123) *ToxB1* is identical to *Pb*(SM106) *ToxB1*; thus, results obtained from analysis of heterologously expressed *Pb*(Bf-1) *ToxB1* and *Pb*(SM106) *ToxB1* should be applicable to these other proteins.

The protein products encoded by selected *ToxB* homologs are shown on the gel in Figure 2B. Because there is a small minority of high molecular weight *Pichia pastoris* proteins present in the culture filtrates of heterologously expressed *ToxB* homologs, traditional quantitation methods to normalize protein concentrations could not be employed (data not shown). Therefore, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for densitometric quantitation of each *ToxB* protein sample (loaded in duplicate) compared with a standard curve of known amounts of homogeneous native *Ptr ToxB*. Heterologously expressed *Pb ToxB* proteins were of similar size to *Ptr ToxB*, which is 6.5 kDa. Samples containing heterologously expressed *Ptr toxb* protein contain three bands, one similar in size to *Ptr ToxB* and a slightly larger doublet (Fig. 3A). All three *Ptr toxb* bands are recognized by *Ptr ToxB* antisera (Fig. 3B), although at a lower affinity than for *Ptr ToxB* (Figs. 3B [prolonged exposure time] and 4). For the purposes of densitometric quantitation, these bands were purposely not separated so that all forms of *Ptr toxb* would be represented in a single band (Fig. 2B).

Heterologously expressed *Pb ToxB* proteins are recognized by anti-*Ptr ToxB* antisera.

Western analysis was performed to determine the affinity of anti-*Ptr ToxB* antisera for the various *Pb ToxB* proteins (Fig. 4). Initially, we loaded equal amounts of all of the heterologously expressed proteins; however, the *Ptr ToxB* recognition signal overwhelmed the signal of any of the other proteins (data not shown). Therefore, we subsequently loaded 10-fold more of each *Pb ToxB* (750 ng) and of *Ptr toxb* (750 ng) compared with *Ptr ToxB* (75 ng). The anti-*Ptr ToxB* antisera recognized *Pb ToxB* and *Ptr toxb*, although with at least 10-fold less affinity than for *Ptr ToxB*, as shown by a similar signal for the

Fig. 3. Culture filtrates produced by heterologous expression of *tox*B contain three protein bands recognized by anti-*Ptr ToxB* antisera. **A**, Culture filtrates containing *Ptr ToxB* (150 ng), *Ptr toxb* (150 ng), and vector alone (control) were resolved by 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were detected by silver stain. **B**, Western blot analysis of heterologously expressed *Ptr ToxB* (75 ng), *Ptr toxb* (750 ng), and vector alone (control) with anti-*Ptr ToxB* antisera. Detection of all three *Ptr toxb* bands required a prolonged exposure time.

Pb ToxB proteins and less intense signal for *Ptr toxb* despite loading 10-fold more *Pb ToxB* and *Ptr toxb* on the gel (Fig. 4). *Ptr toxb* signal under these conditions is very weak; a prolonged exposure is required to observe the second set of bands (data not shown), observed in Figure 3B. Finally, culture filtrate of our vector-alone control was not cross-reactive with the *Ptr ToxB* antisera (Figs. 3B and 4, control), ruling out nonspecific interactions of anti-*Ptr ToxB* antisera with *Pichia* proteins.

Pb ToxB had no effect on tested bromegrass, despite the high susceptibility of bromegrass to *Pyrenophora bromi*.

If *Pb ToxB* functions as an HST in the interaction between *P. bromi* and bromegrass, infiltration of *Pb ToxB* proteins alone would be expected to reproduce disease symptoms on correspondingly susceptible host plants. Concentrations for infiltration of 9.5 ng/μl (1.4 μM), 19 ng/μl (2.88 μM), and 38 ng/μl (5.76 μM) were chosen based on the physiologically relevant concentrations previously published in the *P. tritici-repentis*–wheat interaction literature for *Ptr ToxB* purified from crude culture filtrates (Strelkov et al. 1999) and for heterologously expressed *Ptr ToxB* (Martinez et al. 2001). Infiltration of heterologously expressed *Pb ToxB* proteins into three bromegrass cultivars (Baylor, PL-BDR1, and a commercial cultivar) at the highest concentration chosen for infiltration (38 ng/μl) failed to elicit symptoms on bromegrass (Fig. 5A, representative bromegrass leaf infiltrated with *Pb ToxB*), despite the fact that these are all highly susceptible to *P. bromi* upon inoculation (Fig. 5B, representative bromegrass leaf inoculated with *P. bromi*).

Because the *P. bromi* isolates SM101 and SM106 each contain two different *Pb ToxB* 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*[SM101] *ToxB1* and *Pb*[SM101] *ToxB2*) or SM106 (*Pb*[SM106] *ToxB1* and *Pb*[SM106] *ToxB2*) were mixed and co-infiltrated into bromegrass; however, there still was no effect (data not shown). *Ptr ToxB*, *Ptr toxb*, and the vector-only control also did not cause any reactions on bromegrass (Fig. 5A). Our results indicate that *Pb ToxB* does not function as an HST in brownspot of bromegrass caused by *P. bromi*, at least among the isolate–cultivar combinations we tested.

The *Pb ToxB* proteins are toxic to *Ptr ToxB*-sensitive wheat but not insensitive wheat.

To investigate whether *Pb ToxB* is toxic to wheat, culture filtrates of heterologously expressed *Pb ToxB* proteins were

Fig. 4. Heterologously expressed *Pb ToxB* and *Ptr toxb* are recognized by *Ptr ToxB* antisera, although with lower affinity than for *Ptr ToxB*. Culture filtrates containing 750 ng of each *Pb ToxB* protein and of *Ptr toxb*, as well as 75 ng of *Ptr ToxB*, were separated by 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blot analysis with anti-*Ptr ToxB* antisera. Control = culture filtrate from a *Pichia pastoris* clone transformed with the pPICZαB vector alone.

infiltrated into Ptr ToxB-sensitive wheat compared with Ptr ToxB, Ptr *tox*b, and a vector-only control (Fig. 5C, wheat line 6B662). As expected, infiltration of Ptr ToxB resulted in characteristic chlorosis, the intensity of which increased with increasing concentrations of toxin applied. Additionally, no symptoms at any of the three concentrations applied were elicited from infiltration of Ptr *tox*b or the control. However, a range of symptoms was elicited on Ptr ToxB-sensitive wheat upon infiltration of the various Pb ToxB proteins. Pb(Bf-1) ToxB1 from *P. bromi* isolate Bf-1 gave no reaction at any concentration. Pb(SM106) ToxB1 from SM106 only caused chlorosis at the highest concentration, 38 ng/μl. Pb(SM106) ToxB2 from SM106 and Pb(SM101) ToxB2 from SM101 gave hints of chlorosis at 9.5 ng/μl, which intensified at the higher concentrations of 19 and 38 ng/μl but never reached the levels of chlorosis caused by Ptr ToxB. Finally, Pb(SM101) ToxB1 from isolate SM101 produced symptoms similar to Ptr ToxB at all three concentrations. Based on the phylogenetic relationships between the Pb ToxB proteins (Fig. 2A), Pb(Bf-1) ToxB2, the other Pb ToxB from Bf-1, and Pb(TAM115) ToxB1, the only locus identified in *P. bromi* isolate TAM115, are expected to be inactive like Pb(Bf-1) ToxB1. Additionally, Pb(TW123)

ToxB1, the only locus identified in *P. bromi* isolate TW123, is expected to cause chlorosis only at the highest concentration tested, similar to Pb(SM106) ToxB1. Similar results were also obtained for infiltration of culture filtrates of all of the heterologous proteins into the Ptr ToxB-sensitive wheat cv. Katepwa (data not shown). Additionally, none of the Pb ToxB proteins elicited chlorosis on the insensitive cv. Auburn (data not shown). Thus, the observed activity of Pb ToxB on wheat appears to be cultivar-specific like Ptr ToxB.

***P. bromi* shares a similar phenotype with nonpathogenic *P. tritici-repentis* race 4 isolates in its interaction with Ptr ToxB-sensitive wheat.**

Due to the observation that some of the Pb ToxB proteins are toxic to wheat, we next determined whether isolates of *P. bromi* cause disease on wheat. Although various Pb ToxB proteins are capable of eliciting chlorosis upon infiltration (Fig. 5C), inoculation of Ptr ToxB-sensitive wheat with *P. bromi* isolates carrying the corresponding *Pb ToxB* genes does not result in symptom development (Fig. 5B, representative wheat leaves inoculated with *P. bromi* and a *P. tritici-repentis* race 5 isolate). Instead, all *P. bromi* isolates tested (Fig. 5B, isolate SM101;

Fig. 5. Pb ToxB proteins can elicit chlorosis on Ptr ToxB-sensitive wheat, although lack any phytotoxic activity on smooth bromegrass susceptible to *Pyrenophora bromi*. **A**, Infiltration of culture filtrates containing heterologously expressed Pb ToxB, Ptr ToxB, and Ptr *tox*b into bromegrass. Infiltrations were done at a concentration of 38 ng/μl. One representative leaf of each infiltration is shown. **B**, Inoculation of Ptr ToxB-sensitive wheat (here, the wheat line 6B662) and bromegrass with *P. bromi* (which possesses two copies of *Pb ToxB*), *P. tritici-repentis* race 5 (which possesses 8 to 10 copies of *ToxB*), and *P. tritici-repentis* race 4 (which possesses 1 copy of *tox*b). **C**, Infiltration of heterologously expressed Pb ToxB, Ptr ToxB, and Ptr *tox*b into the Ptr ToxB-sensitive wheat cultivar 6B662. Infiltrations were done at a range of concentrations, including: 9.5, 19, and 38 ng/μl. Control = culture filtrate from a *Pichia pastoris* clone transformed with the pPICZαB vector alone. Similar results were obtained with the Ptr ToxB-sensitive cv. Katepwa (data not shown).

isolates SM106 and Bf-1, data not shown) elicited a response similar to that caused by the nonpathogenic race 4 isolate of *P. tritici-repentis* on Ptr ToxB-sensitive wheat (Fig. 5B). Neither race 4 nor race 5 isolates of *P. tritici-repentis* exhibited a pathogenic reaction when inoculated on bromegrass (Fig. 5B). As expected, *P. bromi* and *P. tritici-repentis* race 5 exhibited typical disease development on their respective hosts (Fig. 5B).

Pb ToxB is expressed

in Ptr ToxB-sensitive wheat inoculated with *P. bromi*.

One explanation for why our *P. bromi* isolates did not cause disease on Ptr ToxB-sensitive wheat, despite possessing genes that encode for phytotoxic proteins, is that the genes are not expressed, or are not expressed to sufficient levels, in the host. Thus, we used semiquantitative RT-PCR to determine whether *Pb ToxB*, specifically for *P. bromi* isolates SM101 and SM106, is expressed in *P. bromi*-inoculated wheat (Fig. 6A). Furthermore, *Pb ToxB* transcript amounts were compared with the amount of transcript detectable in wheat inoculated with a *P. tritici-repentis* race 5 isolate that causes significant levels of chlorosis (Fig. 6B). We chose glyceraldehyde-3-phosphate dehydrogenase (*gpd*) as a control for fungal biomass within a mix of plant and fungal RNA because of its successful use as a reliable reference gene in previous expression analyses of other fungus–host interactions (Fang and Bidochka 2006; Grell et al. 2005). Additionally, we confirmed that the primers *gpd1* and *gpd2* amplified *gpd* from *P. bromi* and *P. tritici-repentis* genomic DNA at similar levels (data not shown).

For our comparison of in planta *Pb ToxB* expression by *P. bromi* in sensitive wheat to that of *ToxB* by *P. tritici-repentis*, we chose to extract RNA from plants at 2 days postinoculation (dpi) for the following reasons. First, Amaike and associates (2008) recently showed that *ToxB* transcript abundance peaks at 1 to 2 dpi in planta, prior to the onset of plant resistance mechanisms. Thus, detection of *Pb ToxB* mRNA at 2 dpi would place *Pb ToxB* protein production at the right time and right place to cause disease. Second, because it was previously shown that infection by *P. tritici-repentis* proceeds similarly up to 48 h in both susceptible and resistant plants, after which either disease progresses or plant defense responses halt pathogen ingress, respectively (Dushnicky et al. 1998), and that the infection phenotypes on wheat for both *P. tritici-repentis* race 5 and *P. bromi* look similar up to this point before diverging significantly (data not shown), we expect that the fungal bur-

den of *P. tritici-repentis* and *P. bromi* is likely similar up to this point and, therefore, dilution of the fungal signal in a majority of plant transcripts will be comparable between the two. Third, by 2 dpi (on both resistant and susceptible cultivars), 95% of surface conidia have germinated and dehydrated, and their associated RNA degraded (De Wolf et al. 1998; Larez et al. 1986), thereby assuring that all fungal RNA detected originates from viable fungal mass within the plant.

Our results reveal that *Pb ToxB* mRNA is expressed at 2 dpi in wheat inoculated with *P. bromi* (Fig. 6A). The presence of stable *P. bromi* mRNA confirms that *P. bromi* is indeed viable during the first 2 days of its interaction with wheat. Furthermore, noninoculated controls confirm that our *gpd* primers do not recognize plant *gpd* sequences (Fig. 6C); therefore, all *gpd* amplification products are fungal specific. Amplification levels of the *gpd* transcript show that similar amounts of fungal biomass are present for both *P. bromi* (Fig. 6A) and *P. tritici-repentis* (Fig. 6B) at 2 dpi; thus, any differences observed between *Pb ToxB* and *ToxB* transcript amounts should represent quantitative differences between the expression levels of these genes. In other words, *Pb ToxB* is expressed by *P. bromi* in wheat at levels considerably lower (Fig. 6A) than *ToxB* in wheat inoculated with *P. tritici-repentis* (Fig. 6B).

DISCUSSION

We previously reported that homologs of *ToxB* from *P. tritici-repentis* are found in several ascomycetes, including sister species *P. bromi*. Due to the close evolutionary relatedness of *P. tritici-repentis* and *P. bromi*, and that of their grass hosts, we investigated whether the homologs of *ToxB* in *P. bromi* act as HST in the disease interaction between *P. bromi* and bromegrass. Our results reveal that heterologously expressed *Pb ToxB* proteins are phytotoxic to Ptr ToxB-sensitive wheat (Fig. 5C) instead of to bromegrass (Fig. 5A), as we had predicted. Furthermore, we show that the corresponding *Pb ToxB* genes are actively transcribed, both in culture (Fig. 1A) and in *P. bromi*-inoculated wheat (Fig. 6A). Thus, although *P. bromi* possesses the pathogenic potential to cause disease on wheat, it does not. Instead, the interaction phenotype of *P. bromi* with Ptr ToxB-sensitive wheat is more similar to that of nonpathogenic *P. tritici-repentis* race 4 isolates (Fig. 5B).

The fact that *ToxB* appears to confer virulence in a copy-number-dependent manner (Amaike et al. 2008, Ciuffetti et

Fig. 6. *Pb ToxB* is transcribed in Ptr ToxB-sensitive wheat that has been inoculated with *Pyrenophora bromi*, although at levels less than *P. tritici-repentis ToxB*. **A**, In planta semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Pb ToxB* and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) expression in the Ptr ToxB-sensitive wheat line 6B662 and cv. Katepwa inoculated with the *P. bromi* isolates SM101 and SM106. **B**, In planta semiquantitative RT-PCR analysis of *ToxB* and *gpd* expression in 6B662 and Katepwa inoculated with the *P. tritici-repentis* race 5 isolate DW7. **C**, In planta RT-PCR analysis of noninoculated wheat controls. Equal amounts (15 μ l) of MassRuler DNA Ladder, Mix, ready-to-use (MBI Fermentas, Hanover, MD, U.S.A.) were loaded on each agarose gel. Mkr = MassRuler DNA Ladder, *Pb* = *P. bromi*, *Ptr* = *P. tritici-repentis*, Kat = Katepwa.

al. 2010) provides insight into a possible explanation for why *P. bromi* is unable to cause disease on wheat, despite possessing genes encoding proteins that are toxic to wheat. Similar to the low-virulent race 5 isolate of *P. tritici-repentis*, which has only two copies of *ToxB* and a concomitant lower level of gene expression and disease elicitation compared with highly virulent *P. tritici-repentis* race 5 isolates that possess eight to 10 copies of *ToxB* (Amaiike et al. 2008), the *P. bromi* isolates used in this study have two copies of *Pb ToxB* (Andrie et al. 2008). Therefore, the inability of these *P. bromi* isolates to cause disease on wheat may be due to levels of *Pb ToxB* expression below those necessary to produce enough active *Pb ToxB* protein to overcome the onset of plant resistance responses by wheat. Indeed, the results of our semiquantitative RT-PCR studies, which show that phytoactive *Pb ToxB* genes are expressed at much lower levels than *ToxB* both in culture (Fig. 1) and in *Ptr ToxB*-sensitive wheat (Fig. 6), are consistent with this explanation. Further support can be found in the dose dependence of symptoms in our infiltration bioassay (Fig. 5C). Because *P. bromi* is viable in wheat (Fig. 6A), it is plausible that, with the acquisition of higher levels of *Pb ToxB* expression, whether via an increase in promoter strength or an increase in copy number of these genes, it could become a pathogen of wheat.

Indeed, fungal genomes are known for their variability, plasticity and flexibility (Zolan 1995). The widespread presence of transposable elements and other repetitive DNA sequences in fungal genomes provides the raw material necessary for gene duplication and gene shuffling (Thon et al. 2006; Turgeon and Baker 2007). Specifically, the genome of *P. tritici-repentis* has been shown to be highly plastic (Aboukhaddour et al. 2009; Lichter et al. 2002) and the truncated transposon-like sequences associated with *ToxB* have been implicated in the multiplication of this gene (Martinez et al. 2004; Strelkov et al. 2006). Similarly, the *Pb ToxB* loci are associated with unique upstream repetitive insertion-deletion (indel) sequences 25 bp in length (tandemly arrayed as either 25, 50, 75, or 150 bp) (Andrie et al. 2008), which conceivably could provide a means for unequal crossing over and the potential for gene amplification or rearrangement. Furthermore, recent phylogenomic analyses of fungi in the Ascomycota have implicated gene inventory flux (expansion and contraction) as an important virulence-associated process (Powell et al. 2008) and shown that regulatory changes in gene expression are clearly coupled to adaptive changes in fungal lifestyle (Thompson and Rekev 2009; Wohlbach et al. 2009). Therefore, it is conceivable that the *P. bromi* genome could change in such a way to increase *Pb ToxB* expression to levels that we believe would be required for *P. bromi* to cause disease on wheat.

We had initially predicted that the variable chlorotic activities (Fig. 5C) arising from the different *Pb ToxB* protein sequences (Andrie et al. 2008) would provide us an obvious window into the active amino acid residues of *Ptr ToxB*; however, a detailed analysis of these data revealed the situation to be more enigmatic than predicted. First, 12 of the 15 amino acid differences between *Ptr ToxB* and the *Pb ToxB* sequences are shared by all of the *Pb ToxB* proteins regardless of their chlorosis-inducing activity on wheat or the lack thereof. Therefore, these residues provide no obvious insight into the residues important for *Ptr ToxB* activity. Second, a straightforward interpretation of two of the remaining three amino acid differences between *Ptr ToxB* and the *Pb ToxB* sequences is confounded when *Ptr toxb*, which is inactive, is also included in the analysis. Specifically, at position 34, *Ptr ToxB* has a glutamic acid (E), functional *Pb ToxB* proteins have a glutamine (Q), and nonfunctional *Pb ToxB* proteins have an arginine (R) (Andrie et al. 2008). Although a change from E to Q is predicted as a

likely substitution and E to R is not (Henikoff and Henikoff 1992), which correlates well with activity versus inactivity of the various *Pb ToxB* proteins, inactive *Ptr toxb* also shares an identical residue (E) in this position with *Ptr ToxB*. Furthermore, in another apparent contradiction, the most highly active *Pb ToxB*, *Pb(SM101) ToxB1*, and the inactive *Ptr toxb* both share a leucine (L) with *Ptr ToxB* at position 59 (Andrie et al. 2008). These observations have led us to conclude that the activity of *Ptr ToxB* is more complicated than just being attributable to single amino acid residues; rather, it possibly involves the formation of proper three-dimensional conformation or distant residues being brought together in the final protein conformation, analogous to signal patches involved in protein sorting (Pfeffer and Rothman 1987). Despite the aforementioned sequence enigmas, the greater affinity of the anti-*Ptr ToxB* antibody for the various *Pb ToxB* proteins compared with its reduced affinity for *Ptr toxb* (Fig. 4) provides support that the *Pb ToxB* proteins and *Ptr ToxB* are more similar to each other than either is to *Ptr toxb*. To delineate which areas of the *Ptr ToxB* protein sequence are important to function, we are taking a domain-swapping approach between *Ptr ToxB*, *Ptr toxb*, and various *Pb ToxB* proteins.

The fact that *Pb ToxB* does not function as an HST in the interaction between *P. bromi* and bromegrass does not preclude that a phytotoxin is involved in brownspot of bromegrass disease. First, many members of the Pleosporales, the order to which *P. bromi* belongs (Eriksson 2005; Schoch et al. 2006), are known to produce HST (Friesen et al. 2008; Walton 1996; Wolpert et al. 2002) or have been shown to produce toxic culture filtrates that correlate with disease symptoms (Strange 2007). Second, in addition to the HST produced by *P. tritici-repentis*, a diversity of phytotoxic metabolites has been specifically described for other grass pathogens in the genus *Pyrenophora*. Three toxins whose activity correlate with disease have been isolated from culture filtrates of *Pyrenophora teres*, causal agent of net blotch of barley, two of which are secondary metabolites (Weiergang et al. 2002) and one of which is proteinaceous (Sarpeleh et al. 2007). *Pyrenophora 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, *Pyrenophora semeniperda* culture filtrates contain metabolites that are selectively toxic to susceptible hosts, although the nature of the active compounds 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 the analysis done for the identification of other phytotoxins. Alternatively, the advent of low-cost, high-throughput, DNA-sequencing technologies might make large-scale functional genomics the approach of choice (Soanes et al. 2007; Xu et al. 2006) to unravel the interaction of *P. bromi* with both wheat and bromegrass.

Our results suggest that there is a potential for *P. bromi* to become a pathogen of wheat. Although there is no current indication in the literature that *P. bromi* associates with wheat, it has been identified in association with other grasses (Sampson and Watson 1985). Furthermore, because *P. bromi* and non-pathogenic *P. tritici-repentis* look nearly identical and suffer misidentifications (Ali and Francel 2003; De Wolf et al. 1998), there is likely a higher incidence of *P. bromi* on wheat than we currently suspect. Together, this points to a need to revitalize our efforts to survey for *P. bromi* on wheat, other cereals, and non-cereal grasses and continue our investigations to elucidate the function of *ToxB* and its homologs, all in an effort to halt the development of an emergent disease.

MATERIALS AND METHODS

Fungal isolates and plant material.

The isolates used in this study included the *P. tritici-repentis* race 4 isolate SD20 (Martinez et al. 2004); the *P. tritici-repentis* race 5 isolate DW7 (Ali et al. 1999); and the *P. bromi* isolates SM101, SM106, and Bf-1 (Andrie et al. 2008). Culture conditions are published elsewhere (Andrie et al. 2007). The smooth brome-grass cultivars used in this study included the line PL-BDR1 (Berg et al. 1989) and cv. Baylor, both obtained from the U.S. National Plant Germplasm System, United States Department of Agriculture–Agricultural Research Service, Western Regional PI Station (Pullman, WA, U.S.A.), as well as a commercial cultivar obtained from Outsidepride.com, Inc. (Independence, OR, U.S.A.). For wheat, the Ptr ToxB-sensitive cv. Katepwa and line 6B662 (Lamari et al. 1995) and the resistant cv. Auburn (Andrie et al. 2007; Ciuffetti and Tuori 1999; Tomas and Bockus 1987; Tomas et al. 1990; Tuori et al. 1995) were used. Prior to inoculation or infiltration, smooth brome-grass was grown in a peat-vermiculite (1:1) mix for 4 to 5 weeks and wheat was grown in soil for 2 to 3 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 brome-grass and wheat were 1.25×10^3 conidia/ml (Zeiders and Sherwood 1986) and 3×10^3 conidia/ml (Lamari and Bernier 1989), 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 approximately 20 to 22°C, and returned to diurnal conditions. Disease development was subsequently monitored and secondary or tertiary leaves for wheat and brome-grass, respectively, were harvested at 5 to 8 dpi to document the infection phenotype. Inoculations were repeated at least two to four times with similar results.

RNA extraction and DNase treatment.

RNA was extracted per a modification of the manufacturer's instructions for the RNeasy plant mini kit (Qiagen, Valencia, CA, U.S.A.) from fungal tissue grown in modified Fries medium (Tomas and Bockus 1987) at 25°C under constant illumination for 10 days (i.e., in culture) or from fungal infected leaf tissue of the wheat cv. Katepwa or line 6B662 harvested at 2 dpi (i.e., in planta). Either type of tissue (200 mg) was ground in liquid nitrogen followed by the immediate addition of 900 µl of RNeasy lysis buffer RLT and incubation at 56°C for 2 min with constant mixing. After vortexing for 30 s, 750 µl was transferred to a QIAshredder minispin column for homogenization, and 450 µl of the flow-through was processed as described by the manufacturer's instructions, including the optional on-column DNase digestion with the RNase-Free DNase set (Qiagen). Further DNA removal was achieved by treatment with TURBO DNase as described for the TURBO DNA-free kit (Ambion, Austin, TX, U.S.A.). RNA samples were diluted to 9 µg per 40 µl prior to treatment, and two applications of TURBO DNase were applied followed by a 30-min incubation at 37°C each time. Prior to subsequent analyses, all RNA samples were screened by PCR with *ToxB*-specific primers for contamination with genomic DNA.

Semiquantitative RT-PCR.

For each sample, first-strand cDNA was synthesized from 1.5 µg of total RNA as described in the manufacturer's instructions for SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Two cDNA reactions of each type were subsequently combined (for a total of 3 µg of RNA per sam-

ple), treated with RNase, and concentrated on a MinElute spin column (Qiagen), which served to remove degraded RNA, as well as the poly-dT primer (as was done by Martinez and colleagues [2004]). PCR was then used to analyze the following: i) 10% of the combined cDNA reaction and ii) 300 ng of the original RNA sample, which corresponds to the total amount of RNA found in 10% of the combined cDNA reaction and serves as a control to rule out contamination with genomic DNA. RNA-only controls were free of contaminating genomic DNA for all sample sets (data not shown).

Primer TB7F (which is identical to *ToxB* but has one nucleotide difference with all of the *Pb ToxB* loci) (Martinez et al. 2001) and TB59R (which is identical to all of the *Pb ToxB* loci but has one nucleotide difference with *ToxB*) (Martinez et al. 2004) were used to detect a 193- and 196-bp fragment of *ToxB* and *Pb ToxB*, respectively. Before using this primer set in reactions with cDNA, it was shown to amplify similarly from equivalent amounts of *P. tritici-repentis* and *P. bromi* genomic DNA (data not shown). Primers *gpd1* and *gpd2* (Berbee et al. 1999), which were designed to amplify an approximately 600-bp fragment from genomic DNA (exon + intron sequence), were used to detect an approximately 450-bp fragment from cDNA (just exon sequence) of the *gpd* gene, our housekeeping gene of choice (Fang and Bidochka 2006; Grell et al. 2005). This primer set was also shown to amplify similarly from equivalent amounts of *P. tritici-repentis* and *P. bromi* genomic DNA (data not shown). Noninoculated controls confirm that our *gpd* primers are specific to fungi and do not recognize plant *gpd* sequences (Fig. 5C). Genomic DNA extraction procedures were previously described (Andrie et al. 2007).

PCR reactions (50 µl) consisted of 1× *Taq* polymerase buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 mM Tris-HCl, pH 9.0), 200 µM each dNTP, 200 nM each primer, and 2.5 U of *Taq* polymerase (MBI Fermentas, Hanover, MD, U.S.A.). 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 35 s, and 72°C for 55 s; and 1 cycle of 72°C for 7 min. To maximize detection of potentially rare *Pb ToxB* transcripts, the amplification products from two PCR reactions were combined, treated with RNase, and concentrated prior to visualization via standard agarose gel electrophoresis. Samples with *ToxB* were treated identically. The identity of all amplification products was confirmed via sequence analysis (Central Services Laboratory, Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR, U.S.A.). PCR reactions were repeated at least twice in culture for each isolate analyzed, as well as 6 times in *P. tritici-repentis*-inoculated wheat and 10 times in *P. bromi*-inoculated wheat.

Heterologous expression in *Pichia pastoris*.

Constructs for heterologous expression in *Pichia pastoris* were assembled in accordance with the instructions for the EasySelect *Pichia* expression kit (Invitrogen). The α -factor signal sequence from *Saccharomyces cerevisiae* present in vector pPICZ α B was chosen for heterologous expression based on preliminary results that revealed superior expression from this signal sequence compared with the native signal sequence (data not shown). Platinum *Taq* DNA polymerase high fidelity (Invitrogen) was used per the manufacturer's instructions to amplify each mature protein sequence without its signal sequence. For use in subsequent cloning steps, the primers PbTB1F (5'-TAGCTCGAGAAAAGAACTGCATCGCCAA TATC-3') and TB16R (Martinez et al. 2001) introduced a 5' *Xho*I site and 3' *Not*I site, respectively, in each amplification product. Gel-purified (Qiagen) amplification products were subsequently digested with *Xho*I and *Not*I and cloned flush with the Kex2 cleavage site of expression vector pPICZ α B,

followed by transformation into *Escherichia coli* XL1-Blue competent cells (Stratagene, La Jolla, CA, U.S.A.). Positive clones for each coding sequence were confirmed via sequence analysis. Constructs were subsequently linearized with *SacI* for transformation into *Pichia pastoris* X-33 competent cells as specified in the instructions for the *Pichia* EasyComp kit (Invitrogen).

For heterologous expression, positive *Pichia pastoris* clones were grown in buffered minimal methanol media as specified by the kit. Each culture filtrate was lyophilized, resuspended in water to approximately $1/10$ the original volume, and desalted on a PD-10 desalting column per the manufacturer's instructions (GE Healthcare, Piscataway, NJ, U.S.A.). Multiple unsuccessful attempts were made to remove the large *Pichia* proteins present in all samples (data not shown); therefore, we relied on culture filtrate from *Pichia pastoris* X-33 transformed with the pPICZ α vector alone for a negative control.

Detection and quantitation of heterologously expressed proteins.

Heterologously expressed protein samples were fractionated by 13% SDS-PAGE in conjunction with the buffer system of Fling and Gregerson (1986) and detected with the SilverSNAP stain kit II (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.). Comparison of the protein products to the MultiMark Multi-Colored Standard (Invitrogen) confirmed that each was approximately 6.5 kDa in size (Fig. 2B), except Ptr toxB, which had an additional, larger doublet (Fig. 3A). To estimate the amount of heterologously expressed protein of interest in each culture filtrate, ImageQuant 5.0 (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.) was used for densitometric analysis of a digital scan of the silver-stained SDS-PAGE gel as described by the manufacturer's instructions. The intensity of two bands for each protein was averaged and compared with a standard curve of pure homogenous native Ptr ToxB standards, including 75, 150, 225, and 350 ng (Fig. 2B). To assist in the quantitation of heterologously expressed Ptr toxB, the two bands seen in Figure 3A were left unresolved in the gel seen in Figure 2B. For leaf infiltration bioassays, the concentration of each heterologous protein was diluted to 9.5, 19, and 38 ng/ μ l, which 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). To rule out an effect of *Pichia pastoris* proteins present in each culture filtrate, we infiltrated the vector-only control at full strength (i.e., it was undiluted as compared with the dilutions described above for the culture filtrates of the heterologous proteins).

Western blot analysis of heterologously expressed proteins.

Protein samples were fractionated as described above, followed by equilibration of the gel in transfer buffer (25 mM Tris Base, 193 mM glycine, and 20% methanol) and dry transfer of proteins to GE nitrocellulose pure transfer membrane (GE Osmonics, Minnetonka, MN, U.S.A.). Resultant blots were blocked overnight at 4°C in 1× TBST (150 mM NaCl, 0.05% Tween 20, and 10 mM Tris-HCL, pH 8.0) and 3% non-fat dried milk. Blots were then washed in 1× TBST, followed by incubation for 1 h in anti-Ptr ToxB antisera diluted 1:2,000 in 1× TBST and 1% bovine serum albumin fraction V. Blots were again washed, followed by incubation for 30 min in anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis) diluted 1:80,000 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 Biotechnology, Inc.). Western analyses were repeated with at least two 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 was documented via digital scanning. Infiltrations were repeated with two to four sets of independently expressed proteins.

ACKNOWLEDGMENTS

We thank S. Ali for isolates of *P. bromi*; P. Martinez for the Ptr ToxB antibody; U.S. National Plant Germplasm System, United States Department of Agriculture-Agricultural Research Service for *Bromus inermis* seed; V. Manning for technical assistance, discussion, and critical review; I. Pandelova for discussion and critical review; and T. Wolpert for critical review. This project was supported by a grant to L. Ciuffetti from the National Research Initiative Biology of Plant-Microbe Associations of the United States Department of Agriculture Cooperative State Research, Education and Extension Service (grant number 2003-35319-13476).

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