Characterization of the Multiple-Copy Host-Selective Toxin Gene, *ToxB*, in Pathogenic and Nonpathogenic Isolates of *Pyrenophora tritici-repentis*

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ToxB, a gene that encodes a 6.6-kDa host-selective toxin (HST), is present in several races of the wheat pathogen *Pyrenophora tritici-repentis*. To learn more about the multiple *ToxB* open reading frames (ORFs), six of the estimated nine copies from a race 5 isolate were cloned and analyzed. All six copies of *ToxB* have identical 261-bp ORFs and thus encode the same form of Ptr *ToxB*. Sequence analysis of regions flanking the cloned *ToxB* loci revealed that the majority of loci are associated with portions of retrotransposons and a transposon-like sequence. Data indicate that *ToxB* loci reside on two chromosomes, 3.5 and 2.7 Mb, with the majority of copies located on the 2.7 Mb chromosome. A related gene, referred to as *toxb*, from a nonpathogenic race 4 isolate was also cloned and characterized. This is interesting because, until now, HST genes have only been found in toxin-producing, pathogenic isolates of plant pathogenic fungi. The *toxb* gene from nonpathogenic isolates is 86% similar to *ToxB*, and data suggest that *toxb* is a single-copy gene. No *toxb* transcript was detected under culture conditions that favor the expression of *ToxB*; therefore, these genes differ in their transcriptional regulation.

Additional keywords: multicopy gene, pathogenicity factor, tan spot.

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs., causal agent of tan spot of wheat, causes significant economic losses in major wheat-growing areas throughout the world. Eight races have been described, based on lesion phenotype on four differential wheat lines (Lamari et al. 1995, 2003; Strelkov et al. 2002). Additional races have been identified, but they have not yet been fully characterized (Ali and Franc 2002; Ali et al. 2003; Manning et al. 2002). Development of disease symptoms from *P. tritici-repentis* involves the production of one to several host-selective toxins (HST) (Ciuffetti and Tuori 1999; DeWolf and Franc 1998; Lamari et al. 2003; Wolpert et al. 2002). The proteinaceous HST Ptr ToxA (Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995; Zhang et al. 1997) [syn. Ptr necrosis toxin, Ptr Toxin, and ToxA (Ciuffetti et al. 1998)] was the first toxin to be fully characterized in *P. tritici-repentis*. The single-copy gene, *ToxA*, encodes the toxin Ptr ToxA, which is present in several races of the tan spot fungus (Ballance et al. 1996; Ciuffetti and Tuori 1996; Ciuffetti et al. 1997). Transformation

of a nonpathogenic isolate with the *ToxA* gene demonstrated that Ptr ToxA functions as a pathogenicity factor in the *Pyrenophora*-wheat interaction (Ciuffetti et al. 1997).

Ptr *ToxB* (syn. Ptr chlorosis toxin) was the second HST to be characterized in *P. tritici-repentis* (Strelkov et al. 1999). Like Ptr ToxA, Ptr *ToxB* is a protein toxin. Additional HST have been identified and partially characterized from *P. tritici-repentis*, including Ptr ToxC (Effertz et al. 2002) and Ptr ToxD (Ali et al. 2002; Manning et al. 2002). It should be noted that preliminary reports by Ali and associates (2002) and Manning and associates (2002) describe two chemically distinct HST that currently share the same name, Ptr ToxD. Race 5 isolates of *P. tritici-repentis*, first found in Algeria, are characterized by the production of lesions with spreading chlorosis on wheat cultivar Katepwa (Orolaza et al. 1995). Race 5 isolates were later identified in North Dakota (Ali et al. 1999). This host-selective chlorosis has been attributed to a 6.6-kDa HST, Ptr *ToxB* (Martinez et al. 2001a; Strelkov et al. 1999). Ptr *ToxB* is encoded by a multiple-copy gene, *ToxB*, comprising a 261-bp open reading frame (ORF) (Martinez et al. 2001a). *ToxB* has recently been found in races 6, 7, and 8, which are all pathogenic on the cultivar Katepwa (Lamari et al. 2003; Strelkov et al. 2002).

Six *ToxB* loci from a race 5 isolate were cloned and characterized to determine if they encode variants of the chlorosis toxin, Ptr *ToxB*. Additional sequences from the flanking regions of the six loci were analyzed for clues about the evolution of this multicopy gene. A *ToxB* related gene, *toxb*, was found in nonpathogenic isolates (Martinez et al. 2001b, 2002). This is interesting because, until now, HST genes have been found exclusively in pathogenic isolates with no corresponding allele or locus in nonpathogenic isolates. To investigate the significance of a toxin-related gene in nonpathogenic isolates, we cloned the *toxb* gene and compared its expression to that of *ToxB* in culture. The chromosomal organization of *ToxB* and *toxb* were also determined by Southern analysis of chromosomes resolved by clamped homogenous electric field (CHEF)-pulsed field gel electrophoresis.

RESULTS

Southern analysis of *ToxB* in various races of *P. tritici-repentis*.

Five genomic *Hind*III fragments from race 5 isolates of *P. tritici-repentis* hybridized to the *ToxB* probe (Fig. 1A). Because *Hind*III does not cut within the 186-bp probe, this result suggests there could be at least five *ToxB* loci in race 5 isolates. However, not all of the *Hind*III fragments hybridized with equal intensity. Assuming that the 1.7-kb band represents

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a single *ToxB* locus, phosphorimage analysis of band intensity relative to the 1.7-kb band indicates that there are nine *ToxB* loci in the race 5 isolates (data not shown). Races 1, 2, 4, and 9 (Manning et al. 2002), known not to produce Ptr ToxB, were also screened for the presence of *ToxB* or a *ToxB*-related sequence. When Southern blots were washed at low stringency, a single band was detected in the race 4 isolate, and no bands were detected in races 1, 2, or 9 (Fig. 1A). When blots were washed at high stringency, the race 4 band becomes very faint relative to the bands in the race 5 isolates (data not shown), thus indicating that the single band in race 4 isolates only shares partial homology to the *ToxB* probe. Consequently, the *ToxB* locus in nonpathogenic race 4 isolates is referred to as *tox*b.

Sequence analysis of the multiple-copy *ToxB* gene from pathogenic race 5 isolates and the related *tox*b gene from nonpathogenic race 4 isolates.

To learn more about the multiple *ToxB* loci and the *tox*b locus, six *ToxB* loci and the *tox*b locus were cloned and sequenced. Analysis of the six *ToxB* loci revealed that all six loci have identical *ToxB* ORFs and, thus, encode the same form of Ptr ToxB. Phosphorimage analysis of band intensity of the Southern blot shown in Figure 1 suggested that there are three to four copies of *ToxB* in the 2.86-kb *Hind*III fragment. Two of the 2.86-kb *ToxB* loci, *ToxB1* and *ToxB2*, were cloned. The

*Hind*III fragments that contain *ToxB1* and *ToxB2* are exactly the same size but differ by a 162-bp inversion (Fig. 1B).

As a result of our cloning strategy, the *ToxB* *Hind*III clones contain only 270 bp of sequence upstream of the *ToxB* ORF, whereas these same clones contain an average of 1,400 bp downstream of the ORF. Analysis of sequence downstream of the *ToxB* ORFs indicated that four of the loci contain coding sequence with significant similarity to known proteins. The two 2.86-kb loci (*ToxB1* and *ToxB2*) and the 2.61-kb locus (*ToxB3*) had a 267-bp region with similarity to a fungal geranylgeranyl pyrophosphate synthetase (accession number P24322, E value = 5×10^{-15}). The 2.86-kb loci contained an additional ORF of 432 bp. When the protein databases were searched with this ORF, 321 of the 432 bp had similarity to a reverse transcriptase found in retrotransposons (accession number AF018033, E value = 0.018) (Fig. 1B, red line). A 400-bp region of the 1.7-kb locus (*ToxB4*) also showed similarity to various plant retrotransposons (accession number AAK02020, E value = 0.004) (Fig. 1B, red line).

To learn more about sequence upstream of the *ToxB* ORFs, a combination of thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR) and PCR with locus-specific primers was employed (Table 1). Analysis of the 5' flanking sequences revealed that at least five of the six *ToxB* loci have a retrotransposon sequence upstream of the ORF (Fig. 1B, red line). This 288-bp retrotransposon-like sequence was most similar to the

Fig. 1. Pathogenic race 5 isolates of *Pyrenophora tritici-repentis* have multiple copies of *ToxB* and race 4 isolates, which are nonpathogenic, have a single copy of a related gene (*tox*b). **A**, *Hind*III-digested genomic DNA of various races of *P. tritici-repentis* was hybridized with a *ToxB* probe. Blots were washed at low stringency to detect *tox*b (race 4), which is 86% similar to *ToxB*. **B**, Diagram of six *ToxB* loci, the *tox*b locus, and their flanking DNA sequences. For the *ToxB* loci, sequence to the right of the dotted line represents data from *ToxB* clones from *Hind*III genomic libraries, except the 1.7-kb locus (*ToxB4*) was cloned by inverse-polymerase chain reaction (I-PCR) of *Hind*III-digested genomic DNA. The size of the *Hind*III clone is indicated next to the locus name. Sequence to the left of the dotted line was obtained by thermal asymmetric interlaced-polymerase chain reaction and PCR with locus-specific primers. Sequence for *tox*b is listed under GenBank accession number AY083456. The accession numbers for the *ToxB* loci and their flanking regions are: *ToxB1*, AY425480; *ToxB2*, AY425481; *ToxB3*, AY425482; *ToxB4*, AY425483; *ToxB5*, AY425484; and *ToxB6*, AY425485.

gypsy-like retrotransposon, *Maggy*, from *Magnaporthe grisea* (accession number AAA33420, E value = 2×10^{-5}). The sequence of the 5' flanking region for the 1.7-kb locus (*ToxB4*) was not determined, because efforts to obtain additional 5' sequence for this locus using TAIL-PCR and inverse (I)-PCR were unsuccessful. At least three of the loci have what appears to be a truncated transposon; that is, 36-bp inverted repeats flanked by 6-bp direct repeats (Fig. 1B, opposing arrowheads). Also, a 197-bp insertion is present upstream of the *ToxB* ORF in the 2.86- (*ToxB1*, *ToxB2*) and 0.93-kb (*ToxB5*) loci. In contrast, this same 197-bp sequence is located downstream of the ORF for the 1.7-kb locus (*ToxB4*) (Fig. 1B, green box).

The *tox*b ORF is 86% similar to *ToxB*, and the predicted protein Ptr *tox*b is one amino acid longer than Ptr *ToxB* and shares 81% identity to Ptr *ToxB* (Fig. 2). Some DNA sequence flanking the *tox*b ORF is also found in most of the *ToxB* loci, but overall, a significant proportion of the *tox*b-flanking sequence is unique to *tox*b (Fig. 1B, orange regions). Most notable are sequence differences upstream of the *ToxB* and *tox*b ORFs that are expected to be within the promoter and enhancer regions. Based on our previous analysis of *ToxB* (Martinez et al. 2001a) in conjunction with partial amino acid sequence of secreted Ptr *ToxB* (Strelkov et al. 1999) and predictions made by the signal peptide prediction program SignalP version 1.1 (Nielsen et al. 1997), it is clear that the first 23 amino acids of Ptr *ToxB* encode a signal peptide. Likewise, SignalP version 1.1 predicts that Ptr *tox*b has a 23-amino acid signal peptide (Fig. 2). Similar to Ptr *ToxB*, Ptr *tox*b has no significant similarity to described proteins in the NCBI databases or to conserved protein motifs in the PROSITE database (Sigrist et al. 2002).

Experiments with *tox*b-specific PCR primers confirmed that *tox*b is absent from race 5 isolate DW7, and similar experiments with *ToxB*-specific primers confirmed that *ToxB* is absent from race 4 isolate SD20 (data not shown).

Chromosome location of the *ToxB* loci and the *tox*b locus.

To determine the genetic organization of the *ToxB* loci and the *tox*b locus at the chromosomal level, chromosomes of race 5 isolate DW7 and nonpathogenic race 4 isolates SD20 and 98-31-2 were resolved on CHEF gels and were analyzed by Southern blot hybridization. When these blots were probed simultaneously with *ToxB* and *tox*b, two prominent bands, 3.5 and 2.7 Mb, were detected in pathogenic isolate DW7 along with several weakly hybridizing bands (Fig. 3B and D). In Figure 3C and D, the 3.5-Mb chromosome comigrates with the 3.1-Mb chromosome, because electrophoretic conditions (method II) used for this gel and the corresponding blot were designed to

resolve chromosomes around 2.7 Mb. As expected, only a single band was detected in nonpathogenic isolates SD20 (3.2 Mb) and 98-31-2 (3.1 Mb) (Fig. 3B). In isolate DW7, the 2.7-Mb band is more intense than the 3.5-Mb band, suggesting that most of the *ToxB* copies are located within the 2.7-Mb band (Fig. 3B and D). Based on a comparison of band intensity of the stained CHEF gel shown in Figure 3A, the 2.7-Mb band appears to represent more than one chromosome. However, a different set of CHEF conditions (method II), designed to better resolve the 2.7-Mb chromosome, suggests that the 2.7-Mb band in DW7 represents a single chromosome (Fig. 3C). Chromosome bands in DW7 other than the 3.5- and 2.7-Mb bands also appear to hybridize to the *ToxB/tox*b probes when blots were washed at high stringency. These may represent truncated *ToxB* sequences. This interpretation is based on comparisons of staining intensity (Fig. 3A) and phosphorimage intensity (Fig. 3B) of the 3.2-Mb *tox*b-containing chromosome in the nonpathogenic race 4 isolate SD20 with the staining intensity and phosphorimage intensity of the weakly hybridizing chromosomes of isolate DW7. The intensity of the *tox*b-containing chromosome in SD20 is expected to be from a single gene copy, and thus, the weakly hybridizing chromosomes in DW7 are less intense than expected for a single copy. No additional chromosomes of isolate DW7 larger than those shown in Figure 3A were resolved under conditions (method III) that resolved chromosomes up to about 4.6 Mb (data not shown). Method III electrophoretic conditions were used to estimate the size of the 3.5-Mb chromosome in isolate DW7.

Expression analysis of *ToxB* and *tox*b.

To examine mRNA expression levels of *ToxB* and *tox*b, isolates DW7 (*ToxB*) and SD20 (*tox*b) were grown in modified Fries medium for 5, 10, and 15 days with constant illumination. DW7 is typically grown under these conditions in our lab for 15 to 18 days for the production of Ptr *ToxB*. Based on

Fig. 2. Alignment of Ptr *ToxB* and Ptr *tox*b from *Pyrenophora tritici-repentis*. 'BLAST 2 Sequences' (Tatiana et al. 1999) was used to align the protein sequences. Identical amino acids are highlighted with black and conserved amino acids are highlighted with gray. The sequence for Ptr *ToxB* (GenBank accession number AY007692) was published previously (Martinez et al. 2001a). Sequence data for Ptr *tox*b are listed under GenBank accession number AY083456.

Table 1. Primers used to obtain additional sequence 5' of the 6 *Hind*III *ToxB* clones.

Primer	Sequence (5' to 3')	Comments
TB3	CTTGATTAGTAAGGCTGAGACCACAGG	Reverse primer for <i>ToxB</i> thermal asymmetric interlaced-polymerase chain reaction (TAIL-PCR)
TB5 ^a	CTACCCACGAATATGCGAAGCTCC	Reverse primer for <i>ToxB</i> TAIL-PCR
TB6 ^a	ACGTCCTCCACTTTGCACACTCTC	Reverse primer for <i>ToxB</i> TAIL-PCR
TB21	ATGAATCCGGGCATGAGGTG	Forward primer for assigning 5' region to <i>ToxB</i> loci
TB22	GCTATCGCCTAAGTTGCACC	Forward primer for assigning 5' region to <i>ToxB</i> loci
TB26	TCTCCATCCTTCTGCCCTTC	Reverse primer specific for <i>ToxB5</i>
TB28	TAACCCAACCCCAACCACAC	Reverse primer specific for <i>ToxB6</i>
TB29	TAGACTCCGCAACAATCAATTC	Part of inverted region between <i>ToxB1</i> and <i>ToxB2</i> ; used as a reverse locus-specific primer for <i>ToxB1</i>
TB32	GGAAGCCCTAGTTAAGACC	Forward primer for assigning 5' region to <i>ToxB</i> loci
TB41	TCCGCAACAATCAATTAAGTG	Part of inverted region between <i>ToxB1</i> and <i>ToxB2</i> ; used as a reverse locus-specific primer for <i>ToxB2</i>
TB42	CCCTTTCCCTTCAGCTTC	Reverse primer specific for <i>ToxB3</i>
TB50	ACCTCGCTACAGCAAAGC	Reverse primer specific for <i>ToxB4</i>
TP1	NGTCGASWGANAWGAA	TAIL-PCR primer used to clone sequence 5' of <i>ToxB</i> ORF

^a Primers also used for applications other than obtaining additional sequence 5' of the *ToxB* clones.

Northern blot analysis of total RNA, it appears that *toxB* is not expressed under culture conditions that favor the expression of *ToxB* (Fig. 4A). However, very low transcript levels often cannot be detected with Northern analysis of total RNA. To maximize detection of rare *toxB* transcripts, we performed reverse-transcriptase (RT)-PCR with platinum *Taq* polymerase high fidelity, and gels were stained with SYBR gold. The RT-PCR experiments did not detect *toxB* mRNA suggesting, that *toxB* is either not transcribed under these conditions or it is transcribed at levels not detectable by our RT-PCR method (Fig. 4B). Similar results were obtained when these experiments were repeated with fungal tissue grown in continuous darkness (data not shown).

DISCUSSION

Genomic organization of *ToxB*.

Phosphorimage analysis suggests there are approximately nine copies of *ToxB* in race 5 isolates of *P. tritici-repentis*. Of these nine copies, six copies were cloned and characterized. *ToxA* and *ToxB*, both from *P. tritici-repentis*, are currently the only characterized genes that directly encode a HST, and only a single *ToxA* gene is found in isolates that produce Ptr ToxA. Other characterized HST are products of multienzymatic pathways (Otani et al. 1995; Walton 1996; Wolpert et al. 2002; Yoder et al. 1997). Some of the nonproteinaceous HST have redundant genes, but not to the extent seen with *ToxB*. *Cochliobolus carbonum*, for example, has three copies of the fatty acid synthase gene *TOXC* and two copies of the peptide synthetase gene *HTSI* (Ahn and Walton 1997; Panaccione et al. 1992). Both genes are required for the production of HC-toxin (Ahn and Walton 1997; Panaccione et al. 1992). *Alternaria alternata* f. sp. *kikuchiana* has multiple copies of several genes involved with the production of AK-toxin (Tanaka and Tsuge 2000; Tanaka et al. 1999); however, gene-knockout experiments suggest that these extra copies are nonfunctional (Tanaka et al. 1999). It is not known if all the *ToxB* loci are actively tran-

scribed or if multiple gene copies result in more transcript, and hence, an increase in disease severity on wheat genotypes that are sensitive to Ptr ToxB. There is some evidence to support this hypothesis. Race 5 isolate 92-171R5 is significantly less pathogenic, that is, it produces less chlorosis than race 5 isolate Alg 3-24 (Strelkov et al. 2002). Isolate 92-171R5 was estimated to have two copies of *ToxB*, whereas Alg 3-24 was estimated to have eight copies (Strelkov et al. 2003). Furthermore, there was less *ToxB* transcript from isolate 92-171R5 grown in culture compared with that from isolate Alg 3-24, although the differences were not quantitated (Strelkov et al. 2003).

The Southern analysis of CHEF-resolved chromosomes suggests that most of the *ToxB* loci are located on a single 2.7-Mb chromosome (Fig. 3B and D). It remains to be determined if these genes are clustered or dispersed.

Comparison of the *ToxB* loci and their flanking regions.

Of the estimated nine copies of *ToxB*, all six copies that we analyzed had identical ORFs and, thus, encode the same form of Ptr ToxB. In addition, the first 400 bp upstream of the translational start site and 250 bp downstream of the translational stop site were identical for five of the six loci. This lack of polymorphism in the *ToxB* ORFs and a portion of the bordering sequence may indicate that the amplification of *ToxB* was relatively recent; and thus, there has not been sufficient time for genetic variation to accumulate. Although the six *ToxB* ORFs were identical, there were differences upstream that could affect the transcription of the various copies. Three of the *ToxB* loci, 2.86 (*ToxB1*, *ToxB2*) and 0.93 kb (*ToxB5*), have a 197-bp insertion located 425 bp upstream of their ORFs

Fig. 3. Identification of chromosomes of *Pyrenophora tritici-repentis* that contain *ToxB* and *toxB*. **A**, Chromosomes of pathogenic race 5 isolate (DW7) and two nonpathogenic race 4 isolates (SD20 and 98-31-2) resolved by clamped homogenous electric field (CHEF) using method I and stained with SYBR gold. **B**, Blot of CHEF gel in panel A probed simultaneously with *ToxB* and *toxB*. **C**, Chromosomes of pathogenic race 5 isolate DW7 resolved by CHEF, using method II and stained with SYBR gold. **D**, Blot of CHEF gel in panel C probed simultaneously with *ToxB* and *toxB*. Arrowheads indicate chromosomes that hybridized strongly to the *ToxB/toxB* probes. Size markers (Mb) are based on chromosomes of *Hansenula wingei*. The size of the 3.5-Mb chromosome in isolate DW7, indicated with an asterisk in panel A, was determined on a separate gel (not shown), using method III electrophoretic conditions and a *Schizosaccharomyces pombe* size standard.

Fig. 4. Nonpathogenic isolate SD20 does not produce a detectable *toxB* transcript under conditions that favor the expression of *ToxB*. **A**, Northern analysis of *ToxB* expression from a pathogenic race 5 isolate (DW7) and *toxB* expression from a nonpathogenic race 4 isolate (SD20). Fungal tissue for both isolates was grown in modified Fries medium for 5, 10, and 15 days with constant illumination. Total RNA (10 µg) was resolved on an agarose gel, was blotted, and was probed simultaneously with *ToxB* and *toxB*. **B**, Reverse-transcriptase-polymerase chain reaction analysis of cDNA from the same RNA samples used in panel A.

(Fig. 1B, green box). The location of this insertion may lie within the promoter of *ToxB*. The *ToxB* promoter is located >300 bp upstream of the ORF, because RT-PCR experiments indicate that the *ToxB* transcript extends at least 305 bp upstream of the ORF (J. P. Martinez, N. W. Oesch, and L. M. Ciuffetti, *unpublished data*). Promoter studies with a reporter gene would provide information about the location and strength of the *ToxB* promoter and the effect of the 197-bp insertion on gene expression.

ToxB has recently been found in races 6, 7, and 8 of *P. tritici-repentis* (Lamari et al. 2003; Strelkov et al. 2002). Strelkov and associates (2003) also reported that race 3 isolate D308 carries the *ToxB* ORF. However, race 3 isolates do not produce chlorosis on the Ptr ToxB-sensitive cultivar Katepwa (Lamari et al. 2003), suggesting that one or more of these copies of *ToxB* are poorly expressed. The *HindIII* restriction-fragment patterns for races 6, 7, and 8 also indicate that *ToxB* is present in multiple copies in these races (Lamari et al. 2003). However, the restriction-fragment patterns were different among these three races, and except for race 6, they were different than the four race 5 isolates that we examined. Our visual examination of the *ToxB* Southern blots of races 6, 7, and 8 from Lamari and associates (2003) also suggests that there are differences in copy number among the races. The polymorphic *ToxB* restriction patterns between the races and the possible differences in copy number suggest that the *ToxB* loci are or were in a state of flux. Direct comparisons between race 5 isolates used in this study and races 3, 6, 7, and 8 isolates were not possible because these isolates were unavailable when these experiments were conducted.

All of the *ToxB* loci were associated with truncated retrotransposon-like sequences (Fig. 1B, red lines). Retrotransposons are one of two classes of mobile elements that are found in all organisms (Berg and Howe 1989). Truncated forms of the original retrotransposon are commonly found in their respective hosts, as was the case for retrotransposon sequences associated with the *ToxB* loci. The retrotransposon sequences associated with the copies of *ToxB* could provide the basis for gene amplification or deletion, or both, by unequal crossing over with the same or similar sequences located elsewhere in the genome. Gao and associates (2002) postulated that the copy number diversity of the *TLH* gene family among *M. grisea* isolates could be explained by unequal crossing over between repetitive elements associated with the *TLH* gene family.

The 288-bp truncated gypsy-like retrotransposon sequence that is found 400 to 600 bp upstream of most of the *ToxB* loci (Fig. 1B, red line) may be involved in gene regulation, because of its proximity to the *ToxB* translational start site. Several mammalian genes are regulated by upstream retroviral sequences (Banville and Boie 1989; Chang-Yeh et al. 1991; Stavenhagen and Robins 1988; Ting et al. 1992). Retrotransposon-like sequences are also expected to regulate several plant genes (White et al. 1994). We were unable to obtain additional 5' sequence for the 1.7-kb locus (*ToxB4*), so it is not known if this locus also has the 288-bp retrotransposon sequence that is associated with the other cloned loci. This 288-bp retrotransposon sequence was most similar to the retrotransposon *Maggy* from *M. grisea* (accession number AAA33420). *Maggy*-like sequences were also found in a race 1 (*ToxB*⁻) isolate of *P. tritici-repentis* (Lichter et al. 2002). *Maggy*-like sequences were present on most of the chromosomes of the race 1 isolate. In contrast, only a few chromosomes of the nonpathogenic race 4 isolate hybridized weakly to the *Maggy*-like probe (Lichter et al. 2002).

Role of *toxb* in nonpathogenic race 4 isolates.

Of the characterized genes involved with the synthesis of HST, there is no corresponding allele found in nonpathogenic

isolates. Thus, we were intrigued to find a gene (*toxb*) in nonpathogenic race 4 isolates that shared 86% similarity to the chlorosis toxin gene, *ToxB*. None of the six cloned *ToxB* loci appear to be allelic with the *toxb* locus. This conclusion is based on the observation that none of the *ToxB* locus-specific regions were found in the *toxb* ORF-flanking regions (Fig. 1B legend). The function of *toxb* in nonpathogenic isolates remains to be determined. If *toxb* is an active gene, it is regulated differently than *ToxB*. *ToxB* transcript was relatively abundant under the tested culture conditions; however, *toxb* transcript was not detected under these same conditions (Fig. 4). This difference in transcription could be a reflection of the significant sequence differences in the first 750 bp upstream of the two ORFs (Fig. 1B). This region most likely contains all or portions of a transcriptional promoter. In contrast to our results, Strelkov and associates (2003) detected a *toxb* transcript by RT-PCR in a race 4 isolate. This discrepancy with our data could be explained by differences in the race 4 isolates. Our *toxb* expression studies involved isolate SD20, and their studies involved isolate 90-2. Alternatively, the contrasting results could be explained by differences in methodology. For example, differences in culture conditions, methods used to synthesize cDNA, or PCR conditions could impact the detection of rare *toxb* transcript. Race 4 isolates, which contain *toxb*, are considered to be nonpathogenic. However, this designation is based on inoculations of wheat differentials tested thus far. These "nonpathogenic" race 4 isolates could be pathogenic on an untested wheat genotype or one or more hosts other than wheat. Interestingly, Ali and Francel (2003) reported that race 4 isolates accounted for 98% of the *P. tritici-repentis* races isolated from noncereal grasses. In contrast, race 4 isolates only composed 5% of the races isolated from bread wheat. Consequently, investigations of the function of *toxb* and its putative protein Ptr toxb should include noncereal hosts that are associated with race 4 isolates. Future studies will be directed toward understanding the function of Ptr toxb in nonpathogenic isolates of *P. tritici-repentis*.

MATERIALS AND METHODS

Fungal isolates.

The following isolates of *P. tritici-repentis* were used in this study. Race 1 isolate BFP (Tuori et al. 1995) is a subculture derived from isolate Pt-1C obtained from W. Bockus (Kansas State University, Manhattan, U.S.A.). Race 2 isolate 86-124 (Lamari and Bernier 1989) was obtained from L. Francel (Pennsylvania State University, University Park, U.S.A.). Race 4 isolate SD20 was obtained from G. Buchenau (South Dakota State University, Brookings, U.S.A.). The four race 5 isolates (DW2, DW7, DW13, and DW16) were collected from durum wheat fields in North Dakota (Ali et al. 1999) and were provided by L. Francel. Race 9 isolate (Manning et al. 2002) SO3 was collected from a wheat field in southern Oregon (M. L. Putnam and L. M. Ciuffetti, *unpublished data*).

Cloning and sequencing of *ToxB* loci and the *toxb* locus.

Three subgenomic *HindIII* libraries were constructed from race 5 isolate DW7. Genomic DNA (5 µg) was digested with *HindIII*, and three fractions (2.5 to 3.1 kb, 1.4 to 1.8 kb, and 0.7 to 1.0 kb) were gel-purified with a Qiagen mini column (Valencia, CA, U.S.A.). Each *HindIII* fraction was ligated with pBS SK+ (Stratagene, La Jolla, CA, U.S.A.), and then, ligated DNA was used to transform ultracompetent XL10-gold *E. coli* cells (Stratagene). The library was screened using standard methods (Sambrook et al. 1989). *ToxB* positive clones were not detected in the 1.4 to 1.8 kb *HindIII* subgenomic library; therefore, inverse (I)-PCR (Ochman et al. 1988; Triglia et al. 1988) was used to clone the 1.7-kb *HindIII* locus (*ToxB4*).

*Hind*III-digested genomic DNA (3 ng/μl) from isolate DW7 was self-ligated. Ligated products were then used in a PCR reaction with primers TB4 (GTGGAGGACGTTGTTAGTAAACAGAG) and TB5 (Table 1). A 1.7-kb I-PCR product was gel-purified and cloned into pGEM-T Easy (Promega, Madison, WI, U.S.A.).

Thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995; Liu et al. 1995) with DW7 genomic DNA was used to obtain sequence information 5' of the *ToxB* ORFs. Table 1 describes primers that were used to obtain this additional 5' sequence. Two TAIL-PCR products, 1.8 and 2.0 kb, were amplified with primers TB3, TB5, TB6, and degenerate primer TP1 and were cloned into the vector pGEM-T Easy. The clones were identical except for a 197-bp insertion in the 2.0-kb clone. To assign the two different 5' ends to some or all of the six *ToxB* *Hind*III loci, PCR experiments were conducted with a forward primer common to the 5' end of both TAIL-PCR products along with a reverse primer specific for a particular locus. For example, primer TB21 (5' end of 1.8- and 2.0-kb TAIL-PCR clones) and primer TB42 (specific for the 2.61-kb *Hind*III locus *ToxB3*) produced a 3.9-kb PCR product from DW7 genomic DNA. This PCR product was sequenced and compared with the 2.61-kb *Hind*III sequence and the two TAIL-PCR products, which allowed assignment of the appropriate 5' region to this particular locus. If a particular locus-specific primer did not produce a product with primer TB21, then primers TB22 and TB32 located 3' of TB21 were used with a locus-specific primer. This approach allowed additional 5' sequence to be assigned for all of the *ToxB* *Hind*III loci except for the 1.7-kb *Hind*III locus (*ToxB4*).

The *ToxB*-like locus in isolate SD20 (race 4) is referred to as *tox*b at this time because this isolate and other race 4 isolates tested thus far are nonpathogenic on wheat genotypes that are sensitive to Ptr ToxB. The *tox*b locus from nonpathogenic isolate SD20 was amplified from genomic DNA with primers TB10 (TATGCGACCCTAACCTAGCC) and TB12 (GCCA-GATAAAAAACCCCTATACC) and cloned into the vector pGEM-T Easy. Additional *tox*b sequence 3' of TB12 was obtained by TAIL-PCR using primers TB9 (CTACAAGCTCCTGTGGTCTC), TB4 (GTGGAGGACGTTGTTAGTAAACAGAG), and degenerate primer AD2 (AGWGNAGWANCAGG). The TAIL-PCR product was cloned into pGEM-T Easy.

To determine if *tox*b was unique to race 4 isolates, PCR reactions were performed with primer TB7 and *tox*b-specific primer TB58 (TATGAATGATTGACTGGGGTTA) and 25 to 50 ng of genomic DNA of isolates DW7 and SD20 (positive control). To determine if *ToxB* was unique to race 5 isolates, PCR reactions were performed with primer TB7 and *ToxB*-specific primer TB60 (ACTAACAACGTCCTCCACTTTG) and 25 to 50 ng of genomic DNA of isolates SD20 and DW7 (positive control). Conditions for PCR were similar to those described for reverse-transcriptase (RT)-PCR.

DNA was sequenced at the Central Services Laboratory, Center for Gene Research and Biotechnology (CGRB), Oregon State University (Corvallis, OR, U.S.A.), on an ABI Prism 3100 Genetic Analyzer using ABI Prism BigDye terminator cycle sequencing (Applied Biosystems, Foster City, CA, U.S.A.).

Southern analysis.

Mycelia for genomic DNA extraction were grown in potato dextrose broth. Lyophilized mycelia (100 to 400 mg) were ground in liquid nitrogen and were added to 7.5 ml of extraction buffer (1% sodium dodecyl sulfate [SDS], 100 mM Na₂SO₃, 100 mM Tris, pH 8.0, 50 mM EDTA). Samples were mixed and incubated at 65°C for 15 to 45 min. After the addition of 3.2 ml of 7.5 M NH₄ acetate, 9 ml of chloroform were

added. Samples were mixed and centrifuged at 10,000 × *g* for 15 min. Nucleic acids from the aqueous phase were precipitated with 1 volume of isopropanol. Nucleic acids were then resuspended in 500 μl 10 mM Tris (pH 8.0), and RNA was digested with 20 μl RNaseA (10 mg/ml). A modification of the method by Michaels and associates (1994) was used to remove most of the polysaccharides. DNA samples were brought to 0.25 M NaCl, and then, an equal volume of 30% ethanol in 0.14 M NaCl was slowly added with continuous mixing. Samples were placed on ice for 10 to 20 min and then were centrifuged at 15,000 × *g* for 10 min. DNA remaining in the supernatant was precipitated with 0.6 volumes of isopropanol. The DNA pellet was washed with 70% ethanol and resuspended in 10 mM Tris (pH 8.0).

Approximately 1 μg of DNA was digested with *Hind*III and was resolved on a 1% agarose gel. DNA was downward transferred onto GeneScreen Plus membranes (Perkin Elmer, Boston). The blot was dried at 80°C and was prehybridized for 1 h at 60°C in 7% SDS, 0.5 M NaHPO₄, and 1% bovine serum albumin. The 186-bp *ToxB* probe was amplified with primers TB6 and TB7 (Martinez et al. 2001a). Following purification of the *ToxB* PCR product on a Qiagen mini column, the PCR product was radiolabeled with α-P³²-dCTP using the Prime-a-Gene random priming kit (Promega). The *ToxB* probe was incubated with the blot for at least 16 h at 60°C. Following hybridization, the blot was washed at low stringency for 5, 10, and 30 min at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS. A Storm PhosphorImager (Amersham, Piscataway, NJ, U.S.A.) located at the CGRB was used to detect DNA fragments that hybridized to the *ToxB* probe.

Northern analysis and RT-PCR.

Tissue for RNA extractions was grown as stationary cultures in modified Fries medium (Tomas and Bockus 1987) at 25°C in the dark or under constant fluorescent light for 5, 10, and 15 days. RNA was extracted from lyophilized mycelia (30 to 45 mg) with Trizol (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions, except an extra chloroform extraction was performed. To help remove polysaccharides, RNA was precipitated with 2 M LiCl for 1 h at −20°C. RNA concentrations were determined using the fluorescent dye RiboGreen (Molecular Probes, Eugene, OR, U.S.A.). Total RNA (10 μg) was resolved according to standard methods and was downward transferred onto a Hybond N+ membrane (Amersham) in 10× SSC. Northern blots were hybridized as described above for Southern analysis with a probe containing equal amounts of *ToxB* and *tox*b. Following hybridization, blots were washed for 10, 10, and 30 min at 55°C in 2× SSC, 0.1% SDS. A phosphorimager was used to detect RNA fragments that hybridized to the *ToxB/tox*b probes.

For RT-PCR, RNA samples were treated with DNase I as specified by the manufacturer of the DNA-free kit (Ambion, Austin, TX, U.S.A.). cDNA was produced as described by Martinez and associates (2001a), except 2 μg of total RNA was used per reaction and oligo-dT primer was removed with a Qiagen MinElute column. RT-PCR reactions (25 μl) consisted of 60 mM Tris-SO₄ (pH 8.9), 18 mM NH₄SO₄, 2 mM MgSO₄, 0.2 mM dNTP, 0.75 units platinum *Taq* polymerase high fidelity (Invitrogen), and 0.2 μM primer TB7 and TB60 for isolate DW7 or TB7 and TB59 (ACTAACAACGTCCTCCA CTTGC) for isolate SD20. PCR reactions were incubated for 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 55°C, and 20 s at 68°C, and a final extension of 5 min at 68°C. To maximize detection of RT-PCR products, agarose gels were stained with SYBR gold (Molecular Probes). Northern analysis and RT-PCR were performed on two independent sets of fungal cultures.

Protoplast preparation and pulsed field gel electrophoresis.

The procedure for generating protoplasts was based on previously published methods (Ciuffetti et al. 1997; Zhong et al. 2002). Protoplast solution (3 to 5 ml per g of mycelium) contained 15 mg of Novozyme per ml (InterSpex, San Mateo, CA, U.S.A.), 5 mg of driselase per ml (Sigma, St. Louis), and 1,500 u of β -glucuronidase per ml (Sigma). For each ml of protoplast solution, the enzymes were first dissolved in 100 μ l 1 M sorbitol before adding 900 μ l of 1.2 M MgSO_4 , 10 mM sodium phosphate buffer (pH 5.8). Mycelia were incubated in protoplast solution for approximately 3 h at 25 to 28°C with gentle shaking. The protoplast suspension was filtered through 100 μ m Nitex (Sefar America, Briarcliff Manor, NY, U.S.A.) and was rinsed with 1 M sorbitol. Protoplasts were pelleted by centrifugation at $2,000 \times g$ for 15 min and were resuspended in 1 M sorbitol. Protoplasts were mixed with molten low melting point agarose (LMA) to achieve 10^8 protoplasts per ml and 0.8% LMA. This mixture was then pipetted into plug molds. Protoplast plugs were incubated overnight in NDS buffer (0.5 M EDTA, 1% sarkosyl, 10 mM Tris, pH 8.0, and 1 mg of proteinase K per ml).

Chromosome-sized DNA from the protoplast plugs was resolved on 0.8 to 1% FastLane agarose (FMC, Rockland, ME, U.S.A.) in $1\times$ Tris-acetate-EDTA buffer at 12 to 14°C with a CHEF DRII system (Bio-Rad, Hercules, CA, U.S.A.). Three sets of running conditions were used. Method I was from Lichter and associates (2002) and consisted of a pulse length of 960 to 480 s over 96 h and an initial voltage of 75 V with a 5 V increase every 24 h. Method II consisted of a pulse length of 720 to 480 s over 72 h and an initial voltage of 80 V with a 5 V increase every 24 h. Method III was based on a modification from Tooley and Carras (1992) and consisted of a pulse length of 3 to 45 min at 45 V for 168 h, with a buffer change after 4 days. Protoplast plugs from *Hansenula wingei* and *Schizosaccharomyces pombe* were used as size standards (Bio-Rad). Chromosome-sized DNA was stained with SYBR gold. CHEF gels were dephurinated and blotted onto Hybond N+ membranes (Amersham) in 0.4 M NaOH. Blots were probed simultaneously with *ToxB* and *toxb*. Following high stringency washes of blots ($0.2\times$ SSC, 0.1% SDS, 60°C), a phosphorimager was used to detect chromosomes that hybridized to the *ToxB/toxb* probes.

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