Research Note

Characterization of the *ToxB* Gene from *Pyrenophora tritici-repentis*

J. Patrick Martinez,¹ Sean A. Ottum,¹ Shaukat Ali,² Leonard J. Franci,² and Lynda M. Ciuffetti¹

¹Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331 U.S.A.; ²Department of Plant Pathology, North Dakota State University, Fargo 58105 U.S.A.

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The *ToxB* gene was cloned and characterized from a race 5 isolate of *Pyrenophora tritici-repentis* from North Dakota. *ToxB* contains a 261-bp open reading frame that encodes a 23 amino acid putative signal peptide and a 64 amino acid host-selective toxin, Ptr ToxB. Analysis of Ptr ToxB from heterologous expression in *Pichia pastoris* confirms that *ToxB* encodes a host-selective toxin.

Additional keywords: pathogenicity, Ptr chlorosis toxin, tan spot, *Triticum aestivum*.

Pyrenophora tritici-repentis, the causal agent of tan spot of wheat, is known to produce multiple host-selective toxins (HSTs) (Ciuffetti and Tuori 1999; De Wolf et al. 1998). Isolates can be categorized as one of five races based on the lesion phenotype on four differential wheat lines (*Triticum aestivum*) (Lamari et al. 1995). Race 1 and 2 isolates produce 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). Transformation of a nonpathogenic isolate with the gene (*ToxA*) for Ptr ToxA clearly demonstrated that Ptr ToxA functions as a primary determinant of pathogenicity in the *Pyrenophora*–wheat interaction (Ciuffetti et al. 1997).

Race 5 isolates, first found in Algeria, are characterized by the production of lesions with spreading chlorosis on cultivar Katepwa (Orolaza et al. 1995). Race 5 isolates later were identified in North Dakota (Ali et al. 1999) based on the infection phenotype on four wheat differentials and, specifically, spreading chlorosis on cultivar Katepwa (Fig. 1A). Strelkov et al. (1999) correlated host-specific chlorosis on Katepwa with a 6.61-kDa HST, Ptr ToxB (syn. Ptr chlorosis toxin) and determined by protein sequence analysis 29 of 30 N-terminal amino acids (aa) of the predicted 63 aa protein.

As a strategy to identify other potential HSTs, we are interested in characterizing isolates of *P. tritici-repentis* that lack the ability to produce either Ptr ToxA or Ptr ToxB, yet are pathogenic on specific wheat cultivars. To facilitate this approach, it was necessary to clone and characterize *ToxB* from a race 5 isolate collected recently in North Dakota (Ali et al. 1999) such that this gene, along with *ToxA*, could be included in a polymerase chain reaction (PCR)-based

screen. Cloning *ToxB* also will facilitate other Ptr ToxB-related research.

The partial protein sequence of Ptr ToxB (Strelkov et al. 1999) was the basis for the reverse transcriptase (RT)-PCR used to amplify an approximately 300-bp fragment from

Fig. 1. Pathogenicity and infiltration assays on four wheat genotypes. **A**, Wheat leaves inoculated with conidia of *Pyrenophora tritici-repentis* isolate DW7 (race 5) on wheat genotypes Katepwa (1), 6B365 (2), Glenlea (3), and Salamouni (4). Inoculation procedures are described in Faris et al. (1997). **B**, Leaves infiltrated with 15 µl of approximately 20 µg of heterologously expressed Ptr ToxB per ml on same wheat genotypes as above (1–4). Katepwa leaf infiltrated with 15 µl of culture filtrate (150 µg of total protein per ml) from nontransformed *Pichia pastoris* (5). Protein concentrations were determined with the DC protein assay (Bio-Rad, Hercules, CA, U.S.A.); lysozyme was used as a standard. Leaves were evaluated for symptoms 72 h after infiltration.

cDNA of isolate DW7 with primers TB2 (5'-ATHAAYGAR-GCNGTIATHGC-3') and CT1 (5'-TTTTGTACAAGCTT₃₀V-N-3'). The procedures for cDNA synthesis and RT-PCR were based on methods described by the manufacturer of Superscript II reverse transcriptase (Life Technologies, Bethesda, MD, U.S.A.), except 5 µl of the first-strand reaction were used in a 50-µl PCR reaction. Sequence analysis confirmed that the approximately 300-bp RT-PCR product encoded part of *Ptr ToxB*. Thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995; Liu et al. 1995) was used to gain an additional 5' and 3' sequence with genomic DNA and the arbitrary primer TP1 (5'-NGTCGASWGANAWGAA-3'). In addition, primers TB3, TB5, and TB6 (Fig. 2) were used for 5' TAIL-PCR and primers TB7, TB8, and TB9 (Fig. 2) were used for 3' TAIL-PCR. Based on the DNA sequence from the 5' and 3' TAIL-PCR products, primers TB10 and TB12 were designed to amplify and sequence a 646-bp fragment from genomic DNA (Fig. 2).

Analysis of the 646-bp fragment revealed a 261-bp open reading frame (ORF) that has no introns and encodes an 87 aa protein with a calculated mass of 8.9 kDa. The ORF shows no significant similarity to sequences in the DNA, protein, or protein motif databases. As expected for a secreted protein, however, the first 23 aa fit the criteria for a signal peptide (Martoglio and Dobberstein 1998). The remaining 64 aa appear to encode mature *Ptr ToxB* (calculated mass of 6.5 kDa), which is similar in size to the 63 aa predicted by Strelkov et al. (1999). In addition to this slight size discrepancy, there also appears to be differences in amino acid composition. Strelkov et al. sequenced 30 N-terminal aa of *Ptr ToxB* from Algerian isolate Alg. 3-24. Our DNA sequence data for mature *Ptr ToxB* from isolate DW7 matches 28 of 30 aa, as reported by Strelkov et al. (1999). The DNA sequence from DW7 encodes a cysteine at aa 2 and 18, reported as an unknown amino acid and a serine, respectively, by Strelkov et al. (1999). Our DNA data was verified by sequence analysis of 19 N-terminal amino acids of *Ptr ToxB* from isolate DW7. Protein for sequence analysis was first obtained by partially purifying culture filtrate with carboxymethyl-Sephadex chromatography, as described by Strelkov et al. (1999). *Ptr ToxB* preparations

were resolved on a denaturing, 10% tricine polyacrylamide gel, electroblotted onto polyvinylidene difluoride membrane, and stained with Brilliant Blue-G colloidal (Sigma, St. Louis, MO, U.S.A.). An approximately 6.6-kDa protein band was excised from the membrane and submitted for sequence analysis (Biotechnology Laboratory, University of Oregon).

Southern analysis suggested that more than one copy of *ToxB* or *ToxB*-like sequences are present in isolate DW7 (data not shown). Therefore, to confirm that the cloned gene encoded *Ptr ToxB*, this gene was expressed heterologously in *Pichia pastoris* in buffered minimal methanol media, in accordance with instructions for the EasySelect *Pichia* expression kit (Invitrogen, Carlsbad, CA, U.S.A.). *P. pastoris* was transformed with vector pCM1 by electroporation. To construct pCM1, primers TB15 (5'-TAGCTCGAGAAAAGAAA-CTGCGTCGCAATATC-3') and TB16 (5'-ATAGTTATGCGGCCGCTATACCTAATGTAGG-3') were used to amplify the sequence encoding the mature 64 aa *Ptr ToxB* protein. The purified PCR product was digested with *XhoI* and *NotI* and cloned flush with the Kex2 cleavage site of the *P. pastoris* expression vector pPICZαB. Culture filtrate from *P. pastoris* transformed with pCM1 was lyophilized, resuspended in water at approximately 1/10 the original volume, and dialyzed overnight at 4°C. Culture filtrate from nontransformed *P. pastoris* for use as a control was processed as described above. *Ptr ToxB* was purified further from *P. pastoris* culture filtrate by cation exchange chromatography with a MonoS HR 5/5 column (Pharmacia, Piscataway, NJ, U.S.A.), as described by Strelkov et al. (1999). Katepwa leaves (susceptible to race 5) became chlorotic when infiltrated with heterologously expressed *Ptr ToxB*, whereas leaves of the three resistant cultivars, Glenlea, 6B365, and Salamouni, were asymptomatic (Fig. 1B). These results are consistent with infiltrations of *Ptr ToxB* purified from Algerian isolates (Strelkov et al. 1999). Unfortunately, we were unable to obtain any of the race 5 Algerian isolates (Orlaza et al. 1995; Strelkov et al. 1999) to compare with our North American race 5 isolate.

Screening large numbers of tan spot isolates for the ability to produce *Ptr ToxA* or *Ptr ToxB* (or lack thereof) can be te-

Fig. 2. Sequence analysis of the *ToxB* gene from *Pyrenophora tritici-repentis*. Potential CAAT motifs and TATA boxes are in bold (Gurr et al. 1987). Arrows indicate the location and sequence of polymerase chain reaction primers. The DNA sequence for a putative signal peptide is underlined (amino acids 1–23). Amino acids 24–87 represent mature *Ptr ToxB*. Asterisks indicate differences in amino acids between our data and the data presented by Strelkov et al. (1999). Nucleotide and amino acid data for *ToxB* have been submitted to GenBank, accession no. AY007692.

dious, and conidial inoculation onto wheat differentials and the isolation of toxins from culture filtrates can be time consuming and laborious. The cloning of *ToxB* will enable us to develop a multiplex-PCR screening approach that may serve as a quick primary screen for *ToxA* and *ToxB* in *P. tritici-repentis*. Unlike the single-copy HST gene *ToxA* (Ballance et al. 1996; Ciuffetti et al. 1997), *ToxB* appears to be present in multiple copies (data not shown). Further investigation of additional *ToxB* loci may provide information about different forms of Ptr ToxB or the evolution of *ToxB*. Heterologous expression of *ToxB* in *P. pastoris* enabled us to demonstrate that the cloned *ToxB* locus encodes a HST. Only Katepwa leaves exhibited typical toxin-induced chlorosis when infiltrated with heterologously expressed Ptr ToxB. Heterologous expression of Ptr ToxB will facilitate additional studies requiring significant quantities of protein such as antibody production, host receptor studies, and crystallography. Given the results presented here and from past studies (Orolaza et al. 1995; Strelkov et al. 1999), it is likely that Ptr ToxB is a primary pathogenicity determinant. This can now be verified by transformation of a *ToxB*⁻ isolate with the *ToxB* gene.

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