A Combination of Phenotypic and Genotypic Characterization Strengthens *Pyrenophora tritici-repentis* Race Identification

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

Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331.

Current address of R. M. Andrie: Division of Infectious Diseases, Department of Medicine, Oregon Health and Science University, Portland 97239.

Accepted for publication 19 December 2006.

ABSTRACT

Andrie, R. M., Pandelova, I., and Ciuffetti, L. M. 2007. A combination of phenotypic and genotypic characterization strengthens *Pyrenophora tritici-repentis* race identification. *Phytopathology* 97:694-701.

Pyrenophora tritici-repentis, causal agent of tan spot of wheat, produces multiple host-selective toxins (HSTs), including Ptr ToxA, Ptr ToxB, and Ptr ToxC. The specific complement of HSTs produced by a particular isolate determines its host cultivar specificity. Each unique specificity profile, represented by the differential induction of necrosis or chlorosis on a standard set of wheat differentials, defines a unique race. Eight races of *P. tritici-repentis* have been formally published, although additional races are under investigation. Although visual assessment of disease phenotype is often used in race designation of *P. tritici-repentis*,

our results suggest that it has the potential to be misleading. Inoculation of the *P. tritici-repentis* isolates SO3 and PT82 on the current wheat differential set indicated classification as race 2 and race 8, respectively; however, genetic characterization revealed that these isolates do not possess the associated HSTs expected for these race assignments. Despite sharing disease phenotypes similar to known races, SO3 and PT82 were genotypically distinct from these previously characterized races of *P. tritici-repentis*. To ensure detection of the breadth of physiological variation among the isolates of *P. tritici-repentis*, our results indicate that race classification, where possible, should include both phenotypic and genotypic analyses and eventual expansion of the differential set.

Additional keywords: host-specific toxins, *ToxA*, *ToxB*.

Pyrenophora tritici-repentis, causal agent of tan spot of wheat, is an economically important pathogen of wheat worldwide (17,20). Methods used to categorize isolates of *P. tritici-repentis* have evolved along with our emerging understanding of the complexity of the interaction between *P. tritici-repentis* and wheat. Prior to the identification of the toxic components produced by *P. tritici-repentis*, Lamari and Bernier (34) developed an isolate categorization system based on a series of lesion types. Resistance was characterized by small, noncoalescing dark brown to black spots, with or without slight amounts of chlorosis or tan necrosis. Moderate resistance to moderate susceptibility was characterized by noncoalescing small dark brown to black spots completely surrounded by a distinct chlorotic or tan necrotic ring. Susceptibility was characterized by chlorotic or tan necrotic lesions, the majority of which coalesce.

Furthermore, isolates were categorized into four pathotypes based on whether they produce necrotic or chlorotic symptoms on distinct wheat cultivars (35). Pathotype 1 isolates produce both necrosis and chlorosis; isolates of pathotype 2 and 3 produce only one symptom each, necrosis or chlorosis, respectively; and non-pathogenic pathotype 4 isolates do not produce either symptom. Of the wheat differentials used to create the pathotype system, cv. Glenlea and the line 6B365 are now used consistently for the detection of necrosis and chlorosis, respectively, when assigning a race designation to a particular isolate (20). It now is known that necrosis on Glenlea can be attributed to production of Ptr ToxA (synonyms: Ptr necrosis toxin, Ptr toxin, ToxA) (9,15,56,57,62), a 13.2-kDa (57) proteinaceous host-selective toxin (HST) encoded

by the *ToxA* gene (10,18). Additionally, Ptr ToxA is responsible for typical necrotic lesions, which often are surrounded by chlorotic halos, associated with tan spot of wheat. Chlorosis on 6B365 can be attributed to production of Ptr ToxC (21), which appears to be a nonionic, polar, low molecular weight HST, the gene(s) for which has yet to be characterized.

The discovery of a distinct chlorotic symptom on wheat cv. Katepwa caused by a previously uncharacterized isolate of *P. tritici-repentis* led to renovation of the pathotype system (37). Lamari and colleagues (37) proposed a race designation system modeled after that of cereal rusts to describe the isolates of *P. tritici-repentis*. The new race-based system is limited only by the size and effectiveness of the differential set, in contrast to the original pathotype classification system that allowed the description of only four pathotypes. The four pathotypes previously described became races 1 through 4, and the new isolate was designated as race 5. Additionally, the wheat line 6B662, formerly considered resistant to *P. tritici-repentis*, was introduced as the differential for race 5. Chlorosis on Katepwa and 6B662 is now known to be due to the production of Ptr ToxB (synonym: Ptr chlorosis toxin) (15), a 6.6-kDa proteinaceous HST (47,52,53) encoded by the *ToxB* gene (43,44). Distinct *ToxB*-like sequences have been identified in isolates of race 3 (50,51) and race 4 (43,51), although neither race produces Ptr ToxB (16,51) or is pathogenic on Ptr ToxB-sensitive wheat (34,37). In race 3, all but the first six nucleotides of the *ToxB*-like sequence are identical to *ToxB* (50,51); whereas, for race 4, the sequences are more divergent (43,50). For example, the *ToxB*-like sequence from the race 4 isolate SD20, designated *toxb*, shares 86% identity with wild-type *ToxB* (43).

The virulence patterns of the various races of *P. tritici-repentis* have since been discovered to be toxin-based, in that each compatible interaction between an isolate and its corresponding susceptible host cultivar is mediated by an HST (51). Therefore,

Corresponding author: L. M. Ciuffetti;

E-mail address: ciuffetl@science.oregonstate.edu

doi:10.1094/PHYTO-97-6-0694

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the specific complement of HSTs produced by a particular isolate determines its race. Ptr ToxA causes necrosis on Glenlea and Katepwa (9,56,57,62), Ptr ToxB causes chlorosis on 6B662 and Katepwa (47,52,53), and Ptr ToxC causes chlorosis on 6B365 (21). Wheat cvs. Salamouni (20,51) and Auburn (18,55-57) are insensitive to all of the characterized HSTs of *P. tritici-repentis*. Isolates producing all possible combinations of Ptr ToxA, Ptr ToxB, and Ptr ToxC have been identified and accordingly are categorized as races 1 to 8 (37,39,51,54). The race structure of *P. tritici-repentis* on the commonly used wheat differential set (34, 37,51) is summarized in Table 1, which indicates the toxin production profiles of each race. Both high- and low-virulence race 5 isolates, as indicated by variable levels of chlorotic symptom development on the Ptr ToxB-sensitive wheat line 6B662, have been identified (54).

Although visual assessment of disease phenotype often is used in race designation of *P. tritici-repentis*, our results suggest that it has the potential to be misleading. Specifically, here we report two examples in which genetic characterization differed from phenotypically determined race designations. Although *P. tritici-repentis* isolate SO3 is known to lack *ToxA* (18), it unexpectedly produced a Ptr ToxA-associated disease phenotype. Additionally, disease phenotype alone led us to mistakenly classify an isolate from Kansas (designated PT82) as race 8 (7), which is indicated by the production of the three characterized HSTs, Ptr ToxA, Ptr ToxB, and Ptr ToxC, although genotypic analysis revealed that it does not possess all of the associated HSTs expected for this race assignment. In documenting these examples, our objective was to demonstrate that accurate race classification based on the current set of published differentials should, where possible, include both phenotypic and genotypic characterization in order to reflect the intimate association between race identity and HST production.

MATERIALS AND METHODS

Fungal isolates. The following two *P. tritici-repentis* isolates were chosen for race characterization: PT82, collected from a field in Kansas, and SO3, collected from a field in southern Oregon (M. L. Putnam and L. M. Ciuffetti, unpublished data).

SO3 originally was designated EO3 and shown to lack the *ToxA* gene via Southern blot analysis (18). However, at the time, inoculation data for all of the differentials were not generated. For comparison, the following *P. tritici-repentis* isolates served as representatives of races 1 through 5: for race 1, isolate SD8; for race 2, isolate 86-124 (35,37); for race 3, isolate D308 (35,37); for race 4, isolate SD20 (43); and, for race 5, isolate DW7 (4). Ideally, isolates of races 6 (54), 7, and 8 (39) also would have been used for comparison; however, because we were unable to obtain these isolates, information presented here on these races is based on published data.

Culture conditions. Cultures of *P. tritici-repentis* were grown on V8 juice agar (20% vol/vol V8 juice, 0.3% wt/vol CaCO₃, and 2% wt/vol Bacto agar) in constant darkness at 25°C. Sporulation of *P. tritici-repentis* was induced by flooding a culture with sterile water and manually depressing aerial hyphae with a glass rod, followed by incubations in 18 to 24 h of light at room temperature and 18 to 24 h of darkness at 16°C (34). To obtain mycelia for DNA extraction, *P. tritici-repentis* conidia were collected in an aqueous solution of 0.015% Tween 20, inoculated into 75 ml of quarter-strength potato dextrose broth (BD Difco, Sparks, MD), and grown at room temperature in a rotary shaker at 175 rpm for 16 h. Mycelia were collected and lyophilized. To obtain crude culture filtrates (CCF) for protein precipitations and toxin identification, three no. 3 borer plugs of V8 agar-grown mycelia were inoculated into 50 ml of modified Fries medium (55), followed by incubation as stationary cultures at 25°C under constant fluorescent light for 19 days.

Plant material and inoculations. The standard wheat differentials included cvs. Glenlea (34), Katepwa (37), and Auburn (18,55,56) and lines 6B662 (37) and 6B365 (34). Plants were grown for 2 to 3 weeks under diurnal conditions of light for 16 h at 23°C and darkness for 8 h at 19°C prior to inoculation. Wheat seedlings were inoculated with a conidial suspension from each isolate (3 × 10³ conidia/ml) to drip stage, incubated overnight in darkness in a dew chamber at ≈20 to 22°C, then returned to diurnal conditions. Disease development was monitored and secondary leaves were harvested 5 to 8 days post inoculation to document infection phenotype. Inoculations were repeated with

TABLE 1. Race structure of *Pyrenophora tritici-repentis*^a

Race	Wheat differentials ^b					
	Glenlea	Katepwa	6B662	6B365	Salamouni	Auburn
1	N (ToxA)	N (ToxA)	R	C (ToxC)	R	R
2	N (ToxA)	N (ToxA)	R	R	R	R
3	R	R ^c	R ^c	C (ToxC)	R	R
4	R	R ^c	R ^c	R	R	R
5	R	C (ToxB)	C (ToxB)	R	R	R
6	R	C (ToxB)	C (ToxB)	C (ToxC)	R	NT
7	N (ToxA)	N (ToxA) C (ToxB)	C (ToxB)	R	R	NT
8	N (ToxA)	N (ToxA) C (ToxB)	C (ToxB)	C (ToxC)	R	NT

^a Based on references 37, 39, and 54.

^b N = necrosis, C = chlorosis, R = resistance, ToxA = presence of *ToxA* and production of Ptr ToxA, ToxB = presence of *ToxB* and production of Ptr ToxB, ToxC = production of Ptr ToxC as evidenced by inoculation, and NT = isolates of race 6, 7, and 8 were not available for inoculation.

^c Although race 3 (50) and race 4 (43,50) possess distinct *ToxB*-like sequences, neither race produces Ptr ToxB (16,50) or is pathogenic on Ptr ToxB-sensitive wheat (34,37).

TABLE 2. Multiplex polymerase chain reaction primers

Locus	Primer	Sequence	Source
<i>ToxA</i>	TA51F	5'-GCGTTCTATCCTCGTACTTC-3'	This study
	TA52R	5'-GCATTCTCCAATTTTCACG-3'	This study
<i>ToxB</i>	TB71F	5'-GCTACTTGCTGTGGCTATC-3'	This study
	TB6R	5'-ACGTCCTCCACTTTGCACACTCTC-3'	43,44
	TB60R	5'-ACTAACAACGTCCTCCACTTTG-3'	43
	TB58R	5'-TATGAATGATTGACTGGGGTTA-3'	43
<i>toxB</i>	CHS-79F	5'-TGGGGCAAGGATGCTTGGGAAGAAG-3'	11
<i>CHS-1</i>	CHS-354R	5'-TGGAAGAACCATCTGTGAGAGTTG-3'	11

similar results at least four times for SO3 and PT82, and at least two times for races 1 to 5.

Southern analyses. DNA was purified by the procedure of Martinez and colleagues (43) via organic extraction in conjunction with a modified polysaccharide precipitation method (46). DNA concentrations were determined by comparison with λ DNA standards on an ethidium bromide-stained 1% agarose gel. Approximately 1 μ g of restriction enzyme-digested genomic DNA was size fractionated followed by downward transfer onto Zeta-Probe Blotting Membranes (Bio-Rad Laboratories, Hercules, CA) via alkaline transfer. For *ToxA*-probed Southern blots, DNA was

digested with either *SacI* or *EcoRV*; for *ToxB*-probed Southern blots, DNA was digested with either *HindIII* or *EcoRV*. Blots were dried at 80°C for 2 h, prehybridized in 7% sodium dodecyl sulfate (SDS), 0.5 M NaHPO₄ (pH 7.2) (a 1 M NaHPO₄ [pH 7.2] stock solution is composed of 71 g of Na₂HPO₄ and 4 ml of 85% H₃PO₄ per liter), 0.001 M EDTA (pH 8.0), 1% bovine serum albumin (BSA) fraction V (14) at 60°C for at least 2 h, and hybridized with labeled probe at 60°C overnight.

Membranes were probed with either a 591-bp fragment of *ToxA* or a 243-bp fragment of *ToxB* obtained with polymerase chain reaction (PCR). Primers TA4bF (5'-GGTCATGCGTTCTATCCT-

Fig. 1. Race designation of *Pyrenophora tritici-repentis* isolates SO3 and PT82 based on phenotypic characterization. **A**, Disease reaction of SO3 on the current set of wheat differentials matches that of race 2 (i.e., Ptr ToxA-induced necrosis on the wheat cvs. Glenlea and Katepwa). **B**, PT82 produces a race 8 disease profile (i.e., Ptr ToxA-induced necrosis on Glenlea and Katepwa, Ptr ToxB-induced chlorosis on Katepwa and 6B662, and Ptr ToxC-induced chlorosis on 6B365) on the current wheat differential set as determined by comparison with disease phenotype standards. Disease phenotype standards represent the disease reaction caused by the production of individual HSTs on corresponding sensitive differentials. These were obtained via inoculation of races that, thus far, have been shown to produce only one toxin each, Ptr ToxA (race 2), Ptr ToxB (race 5), or Ptr ToxC (race 3). Resultant disease phenotypes on corresponding sensitive differentials are assumed to be due to each respective toxin. **C**, Comparison of PT82 and race 1 on the Ptr ToxB-sensitive wheat line 6B662. Res = resistant, 2/ToxA = Race 2/Ptr ToxA, 5/ToxB = Race 5/Ptr ToxB, 3/ToxC = Race 3/Ptr ToxC, Aub = Auburn, Glen = Glenlea, and Kat = Katepwa.

CGTAC-3') and TA14bR (58) were used to amplify the *ToxA* probe. The *ToxB* probe was amplified by primers TB57F (5'-GAGACTGCTATGCTACTTGCTG-3') and TB6R (Table 2) (43, 44). The reaction mixture was composed of 1× *Taq* polymerase buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0), 200 μM each dNTP, 200 nM each primer, 2.5 U of *Taq* polymerase (MBI Fermentas, Hanover, MD), and 10 ng of template DNA in 50 μl. Amplification was carried out as follows: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 7 min. PCR products were purified on MinElute Spin Columns (Qiagen, Valencia, CA) and α-³²P-dCTP-labeled with the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA) per the manufacturer's instructions.

Hybridization was followed by three washes with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS at 60°C for 5, 10, and 30 min, and a final high stringency wash with 0.2× SSC and 2% SDS at 60°C for 45 min. To detect probe hybridization, blots were exposed to autoradiographic film (Denville Scientific, Metuchen, NJ). At least two independent DNA samples for each isolate were analyzed via Southern analysis.

Western analyses. For Western analyses of Ptr ToxA and Ptr ToxB, total proteins were precipitated from 500-μl and 1-ml aliquots of CCF, respectively, via the trichloroacetic acid (TCA) procedure of Ciuffetti and colleagues (18). Protein samples were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the buffer system of Fling and Gregerson (23). SDS-PAGE gels then were equilibrated in transfer buffer (25 mM Tris Base, 193 mM glycine, 20% methanol), followed by dry transfer of proteins to GE Pure Nitrocellulose Transfer Membrane (GE Osmonics, Minnetonka, MN). Resultant blots were blocked overnight at 4°C in 1× TBST (150 mM NaCl, 0.05% Tween 20, 10 mM Tris-HCl, pH 8.0) and 3% nonfat dried milk. Blots then were washed in 1× TBST and incubated for 1 h in either anti-Ptr ToxA N+C fusion protein antisera (41) or anti-Ptr ToxB antisera diluted 1:10,000 or 1:2,000, respectively, in 1× TBST and 1% BSA fraction V. Blots were washed again and incubated for 30 min in anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis) diluted 1:8,000 in 1× TBST. Following a final wash, blots were developed via chemiluminescence with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) per the manufacturer's instructions. Western analyses were repeated on two independent CCF samples for both Ptr ToxA and Ptr ToxB.

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

Primers used for multiplex PCR are listed in Table 2. Primers specific to the chitin synthase 1 gene, *CHS-1* (11), were added as an internal control for the presence of fungal DNA. When used with primer TB71F, primer TB60R (43) amplifies both *ToxB* in race 5 and the *ToxB*-like sequence in race 3, whereas primer TB58R (43) amplifies only *tox*b from race 4. Reaction conditions were as follows: 1× *Taq* polymerase buffer, 200 μM each dNTP, 200 nM each primer, 2% (vol/vol) dimethyl sulfoxide, 1.25 U of *Taq* polymerase, and 10 ng of DNA template in 25 μl. Following an initial 1-min, 94°C denaturation, samples underwent 30 cycles

of 94°C for 45 s, 58°C for 30 s, and 72°C for 1 min, 30 s, ending with a final 7-min extension at 72°C. We repeated multiplex PCR on at least two independent DNA samples.

RESULTS

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

SO3 previously was shown to lack the *ToxA* gene (18). At that time, it was assumed that SO3 was nonpathogenic because of the absence of *ToxA*. In contrast, subsequent inoculations of SO3 on the standard wheat differential set have revealed that it produces a disease phenotype matching that of race 2 (necrotic lesions with chlorotic halos on Glenlea and Katepwa, and a resistant reaction on other tested differentials) (Fig. 1A). The disease phenotype of PT82 did not match any of the disease profiles for races 1 to 5 (data not shown). However, comparison with disease phenotype standards revealed that PT82 shares the Ptr ToxA necrosis phenotype on Glenlea and Katepwa with race 2, the Ptr ToxC chlorosis phenotype on 6B365 with race 3, and the Ptr ToxB chlorosis phenotype on Katepwa and 6B662 with race 5 (Fig. 1B). Although the chlorotic symptoms elicited by PT82 were less severe than those caused by the race 5 isolate DW7, we declared it to be a positive chlorotic reaction because (i) it was within the range of symptoms documented by Strelkov and colleagues (54) for low and high virulence race 5 isolates on 6B662; (ii) a survey of published literature on races 5 (44,52), 6 (54), 7, and 8 (39) revealed that the reaction of 6B662 to Ptr ToxB varies depending on the isolate; and (iii) it was distinctly different than the resistant response observed on the wheat differential Auburn (Fig. 1B, right panel). That PT82 exhibited all three toxin phenotypes led us initially to designate it as race 8, which is characterized by the production of Ptr ToxA, Ptr ToxB, and Ptr ToxC (Table 1).

Genotypic characterization. We utilized a molecular approach to evaluate the phenotypically determined race designations of SO3 as race 2 and PT82 as race 8. The availability of the *ToxA* (10,18) and *ToxB* (44) genes and antibodies (41) to both toxins facilitated Southern and Western analyses, respectively. Because Ptr ToxC has not been completely characterized, tools for genotypic analysis are not available. Hybridization patterns in Southern analyses for races 1 to 5 were as expected. The *ToxA* probe hybridized to *SacI*-digested genomic DNA of races 1 and 2, but not to that of races 3, 4, or 5, whereas the *ToxB* probe hybridized to *HindIII*-digested genomic DNA of races 3, 4, and 5, but not to that of races 1 or 2 (Fig. 2A). Comparable results were obtained for hybridization of either probe to *EcoRV*-digested genomic DNA (data not shown). As shown by Western analyses, all races that contained *ToxA* produced Ptr ToxA; however, not all races with *ToxB* produced Ptr ToxB (Fig. 2B). Although races 3 and 4 possess homologs of *ToxB* (Fig. 2A), they do not produce detectable amounts of Ptr ToxB (Fig. 2B), nor do they infect Ptr ToxB-sensitive wheat differentials (data not shown). As expected, the race 5 isolate produced Ptr ToxB (Fig. 2B).

For both SO3 and PT82, molecular characterization contradicted phenotypic race classification. Although SO3 demonstrated a Ptr ToxA phenotype on Glenlea and Katepwa (Fig. 1A) and mimics a race 2 disease profile, it is known to lack the *ToxA* gene (18). Due to this discrepancy, we reconfirmed the absence of *ToxA* (Fig. 2A) and Ptr ToxA production (Fig. 2B), confirming, in turn, that SO3 clearly is not a race 2 isolate. Consistent with its designation as race 8, PT82 possessed the *ToxA* gene (Fig. 2A) and produced Ptr ToxA (Fig. 2B); however, it lacked the *ToxB* gene (Fig. 2A) and Ptr ToxB production (Fig. 2B), despite causing chlorosis on Katepwa (Fig. 1B) and 6B662 (Fig. 1B and C). Thus, PT82 is not race 8.

Comparison of PT82 and race 1 on the Ptr ToxB-sensitive wheat line 6B662. Due to the lack of the *ToxB* gene and Ptr ToxB production by PT82, we investigated the possibility that the phenotype on 6B662 caused by this isolate resulted from the combined production of Ptr ToxA and Ptr ToxC. To do so, we inoculated 6B662 with a race 1 isolate, which produces both Ptr ToxA and Ptr ToxC, and compared the resultant phenotype with that caused by PT82 (Fig. 1C). In contrast to the chlorotic reaction caused by PT82 on 6B662, race 1 isolate SD8 produced a typical resistant response, which is characterized by distinct, dark-brown lesions. The response of 6B662 to race 1 (Fig. 1C, left panel) is similar to the response of this cultivar to race 2 (Fig. 1A, left panel). Thus, it appears that the response of 6B662 to PT82 inoculation is not due to the combined effect of Ptr ToxA and Ptr ToxC, and also that PT82 is not race 1.

Fig. 2. Genetic characterization of PT82 and SO3 differs from phenotypically determined race designations. **A**, Southern analyses of PT82 and SO3 compared with *Pyrenophora tritici-repentis* races 1 to 5. *SacI*- or *HindIII*-digested genomic DNA (1 µg) from each isolate was hybridized with a *ToxA* or *ToxB* probe, respectively. **B**, Western analyses of PT82 and SO3 compared with *P. tritici-repentis* races 1 to 5. For each isolate, total precipitated protein from 500 µl or 1 ml of crude culture filtrate was subjected to Western analyses by using Ptr ToxA- or Ptr ToxB-specific antibodies, respectively.

Multiplex PCR. To facilitate rapid characterization of *P. tritici-repentis* isolates, a PCR-based approach was developed to simultaneously identify disease determinants whose genes have been characterized. Primer sets were designed to amplify portions of both the *ToxA* and *ToxB* open reading frames (Table 2). The *ToxA* primer set amplified a 573-bp fragment, whereas the *ToxB* primer set amplified a 232-bp fragment (Fig. 3A). Primers (Table 2) for *CHS-1* (11), the gene for chitin synthase, were included as an internal control for fungal DNA and produced a 275-bp amplification product (Fig. 3). These three amplification product sizes were easily distinguished by DNA agarose gel electrophoresis and, thus, proved useful for multiplex PCR.

Multiplex PCR amplification patterns for races 1 to 5 (Fig. 3A) were as expected based on results of Southern analyses (Fig. 2A). Races 1 and 2 yielded amplicons with *ToxA*-specific primers and races 3, 4, and 5 yielded amplicons with *ToxB*-specific primers (Fig. 3A). Two additional primer sets (Table 2) differentiated the *ToxB* sequences in races 3 and 5 from *tox**b*, the *ToxB* homolog in race 4 isolate SD20 (Fig. 3B and C). When paired with the forward primer TB71F, reverse primer TB60R (43) was specific to the *ToxB* sequences in races 3 and 5 (Fig. 3B), whereas reverse primer TB58R was specific to *tox**b* in race 4 (Fig. 3C). Multiplex PCR amplification patterns for SO3 and PT82 (Fig. 3A) also confirmed Southern analyses (Fig. 2A). SO3 tested negative for both genes, whereas PT82 tested positive for *ToxA* but not *ToxB*. A *CHS* amplification product was detected in every PCR reaction that contained fungal DNA (Fig. 3).

DISCUSSION

P. tritici-repentis exhibits a complex race structure in its interaction with wheat (Table 1). Eight races are distinguished by the differential expression of the three currently identified HSTs produced by this fungus, which in turn often are identified by distinct disease reactions on current wheat differentials. Additional races of *P. tritici-repentis* have been suggested (2,5,16, 42,45) but await publication in a peer-reviewed journal. Although *P. tritici-repentis* race classification has been based primarily on disease phenotype, we present two examples where using disease phenotype alone to designate race and associated toxin production led to erroneous assignments. Inoculation of the *P. tritici-repentis* isolates SO3 and PT82 on the standard wheat differential set indicates classification as race 2 and 8, respectively (Fig. 1). However, genetic characterization reveals that these isolates do

Fig. 3. Multiplex polymerase chain reaction (PCR) detection of host-selective toxin genes in isolates of *Pyrenophora tritici-repentis*. **A**, Agarose gel electrophoresis of multiplex PCR amplification products from genomic DNA of SO3, PT82, and races 1 to 5. **B**, Multiplex PCR modified to include *ToxB*-specific primers. **C**, Multiplex PCR modified to include *tox**b*-specific primers. Primers for the chitin synthase 1 gene, *CHS-1*, provide an internal control for the presence of fungal DNA.

not possess all of the associated HSTs expected for these race assignments (Figs. 2 and 3); specifically, SO3 lacks the *ToxA* gene and Ptr ToxA production and PT82 lacks the *ToxB* gene and Ptr ToxB production. Because races of *P. tritici-repentis* are distinguished by the HSTs they produce (51), not just on disease phenotype alone, the fact that SO3 does not possess the *ToxA* gene and PT82 does not possess the *ToxB* gene is evidence enough that these isolates cannot be classified as race 2 or race 8, respectively. Thus, to ensure accurate race classification, our results suggest that genotypic confirmation should accompany, where possible, phenotypic characterization when designating a *P. tritici-repentis* isolate to race.

Inclusion of genotypic characterization also has the potential to eliminate the subjectivity inherent in visual assessments of disease phenotype, as was the case for race classification in *Cochliobolus carbonum*, causal agent of Northern leaf spot and ear rot of maize (*Zea mays* L.). Exclusive of race 1, which is unequivocally identifiable by disease phenotype due to the production of the HST HC-toxin (27,49,59), Jones and Dunkle (31) declared *C. carbonum* race identification based on disease reaction to be ambiguous, faulting both phenotypic variation among different isolate-cultivar combinations and the use of an inconsistent set of inbred lines for race characterization. As such, they examined whether fungal genotyping could be used to discern races of *C. carbonum* and found that PCR amplification with both arbitrary and gene-specific primers is useful for *C. carbonum* race differentiation. A similar potential for subjectivity in the *P. tritici-repentis*-wheat interaction is highlighted by the range of chlorotic symptoms observed on the wheat differential 6B662 in response to infection by various Ptr ToxB-producing isolates. Differences in chlorosis are observed both between isolates of the same race (i.e., low- and high-virulence race 5 isolates) and among isolates of different races (i.e., races 5, 6, 7, and 8) (39,44,54). Because genotypic characterization provides an objective assessment of the presence of an HST and, thus, race identity, it promises to provide similar benefits to *P. tritici-repentis* race classification as were obtained for *C. carbonum*.

Although the identification of previously unidentified phenotype-genotype combinations suggests that we have uncovered two new races of *P. tritici-repentis* that potentially produce two new toxic activities on wheat (for SO3, the necrotic disease reaction on Glenlea and Katepwa [Fig. 1A]; for PT82, the chlorotic disease reaction on Katepwa and 6B662 [Fig. 1B and C]), official race designation of these isolates awaits their thorough characterization. Thus, a priority in our laboratory is to characterize the putative races represented by *P. tritici-repentis* isolates SO3 and PT82 and their associated toxic activities, including the genetic determinants responsible for toxin production. Evidence suggests that the necrotic symptoms produced by SO3 on Glenlea and Katepwa are caused by a previously undescribed proteinaceous HST (48), which we have tentatively named Ptr ToxD (42). It must be noted that an independently identified toxic compound also was simultaneously labeled as Ptr ToxD (5,45), although official designation of either of these toxic activities as an HST awaits formal publication as designated by the criteria for standardized *P. tritici-repentis* HST nomenclature (15). Similar standardization of criteria for *P. tritici-repentis* race designation has yet to be established; however, the tan spot research community is aware of this need and a collaborative publication similar to that for *P. tritici-repentis* HST nomenclature (15) is anticipated.

A logical requirement for designation of a new race is the development of a positive screen for its identification. Ideally, race classification would be based on a standard set of differentials made up of distinct cultivars specific to each unique toxic compound produced by *P. tritici-repentis*. Indeed, identification of a cultivar specific for the toxic compound produced by SO3 is a current effort in our laboratory, although we have yet to identify a cultivar that separates sensitivity to Ptr ToxA from sensitivity to

the toxic compound produced by SO3 (I. Pandelova, O. Riera-Lizarazu, and L. M. Ciuffetti, *unpublished data*). However, implementation of an expanded differential set for race classification in lieu of the inclusion of genotypic characterization deserves careful consideration. First, it appears that two putatively distinct toxic compounds (Ptr ToxA and the putative toxic compound produced by SO3) can cause similar symptoms on the same host genotype. Because this scenario may not be unique, the exclusion of genetic confirmation of phenotypic race characterization could lead to misclassifications similar to those presented here. Second, infinite expansion of the differential set to account for each newly characterized toxic compound eventually could make race classification based on phenotype too cumbersome for individual laboratory implementation (12). The ease with which genotypic characterization can be executed provides yet another reason why it should complement disease phenotype in the classification of *P. tritici-repentis* isolates to race.

Indeed, the versatility of PCR-based methodologies has led to their widespread application to investigations in plant pathology (29). Races of fungal pathogens most commonly are distinguished genotypically by molecular marker methods that employ arbitrary primers, such as restriction fragment length polymorphism (1,32, 40,60), random amplified polymorphic DNA (8,13,19,26,28, 33, 60), and amplified fragment polymorphism (AFLP) assays (63), although some methods utilize specific primers (30,61). Because AFLP analysis did not differentiate between the races of *P. tritici-repentis* (24), we took advantage of the intimate association between HST production and race identity to develop a straightforward and high-throughput multiplex PCR assay based on HST gene-specific primers (Table 2) to aid in race differentiation (Fig. 3).

ToxA and *ToxB* currently are the only HST genes available for use in genetic screens of *P. tritici-repentis*. Due to the worldwide distribution of *P. tritici-repentis* races that possess the *ToxA* or *ToxB* genes (3,6,22,35,36,38,51), our multiplex PCR approach promises to be very useful for characterization of isolates to race. Even so, genotypic race characterization alone cannot yet replace phenotypic race designation. Above all, disease determinants other than Ptr ToxA and Ptr ToxB have not been fully characterized. The most glaring omission from our multiplex PCR assay is a screen for the presence of Ptr ToxC. The gene or genes involved in the synthesis of Ptr ToxC, a disease determinant produced by the *P. tritici-repentis* races 1, 3, 6, and 8 (Table 1), have yet to be cloned. Because race 1 isolates are prevalent throughout the world (3,6,22,35,36,38,51) and race 3 isolates have been found in North America (22,51), Europe (6), and Asia (38,51), Ptr ToxC is the leading candidate for inclusion into the multiplex PCR approach.

The presence of *ToxB* homologs in races that are not pathogenic on Ptr ToxB-sensitive wheat, in particular in race 3 (50) and race 4 (43,50), also confounds exclusive use of our multiplex PCR to designate race. Although nonpathogenic race 4 isolates can be identified with our *tox*-specific primers (Fig. 3C), our *ToxB*-specific primers recognize both *ToxB* in race 5 and its homolog in race 3 isolates (Fig. 3B). Because race 3 isolates do not produce Ptr ToxB (Fig. 2B), it follows that all positive multiplex PCR results obtained with our *ToxB*-specific primer set require direct isolation of the toxin from crude culture filtrate or confirmation of Ptr ToxB expression via Western analysis (Fig. 2B). Even so, multiplex PCR serves to limit the number of isolates that necessitate these more laborious manipulations. Alternatively, development of a race 3-specific primer set could alleviate this concern.

Our results reveal that a combination of phenotypic and genotypic characterization should be used for *P. tritici-repentis* race designation. Despite the production of characterized disease phenotypes by *P. tritici-repentis* isolates SO3 and PT82, genotypic characterization revealed the absence of the HSTs suggested by inoculation of the differential set. The identification of previously unidentified phenotype-genotype combinations suggests

that these isolates represent two new races of *P. tritici-repentis* that potentially produce two new toxic compounds on wheat. However, these await thorough characterization. The likely discovery of new toxic compounds produced by *P. tritici-repentis*, both as suggested here on the current differential set and as predicted on an expanded differential set (12,38), indicates an even more complex race structure for *P. tritici-repentis* than already described. Additionally, in a community effort that currently is in progress in our laboratory in collaboration with the Broad Institute (25), completion of the genomic sequence of a race 1 isolate and the production of EST libraries from additional races promise to significantly increase the discovery rate of pathogenicity or virulence factors produced by this fungus. The complexity of the interaction between *P. tritici-repentis* and wheat emphasizes the need for systematic characterization of the HSTs and races of *P. tritici-repentis*, and for the availability of all published races and differentials for accomplishing this goal. As our understanding of the *P. tritici-repentis*-wheat interaction becomes more defined, additional genotypic tools will become available to evaluate this system.

ACKNOWLEDGMENTS

This work was supported by the National Research Initiative of the United States Department of Agriculture Cooperative State Research Education and Extension Service, grant number 2003-35319-13476. We thank those who contributed isolates of *P. tritici-repentis*, including W. W. Bockus (Kansas State University, Manhattan) for PT82, G. Buchenau (South Dakota State University, Brookings) for SD8 and SD20, L. Lamari (University of Manitoba, Winnipeg, Canada) for 86-124 and D308, and the late L. Francel (Pennsylvania State University, University Park) and S. Ali (North Dakota State University, Fargo) for DW7; R. Tuori for primer TA4bF, J. P. Martinez for primer TB58R and the Ptr ToxB antibody, and J. Spatafora for use of his FastPrep Instrument; V. Manning for technical assistance and critical review; and T. Wolpert and K. Johnson for helpful suggestions and critical review.

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