Identification and Characterization of Novel Isolates of *Pyrenophora tritici-repentis* from Arkansas

Shaukat Ali, Suraj Gurung, and Tika B. Adhikari, Department of Plant Pathology, North Dakota State University, NDSU Dept. 7660, P.O. Box 6050, Fargo, ND 58108

ABSTRACT

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Tan spot, caused by *Pyrenophora tritici-repentis*, is an important foliar disease of wheat (*Triticum aestivum*) worldwide. In a preliminary study, *P. tritici-repentis* isolates from Arkansas were shown to vary in virulence relative to isolates from other regions of the United States. Therefore, the aim of the current study was to characterize both pathogenic and molecular variations in *P. tritici-repentis* isolates from Arkansas. The virulence of 93 isolates of *P. tritici-repentis* was evaluated by inoculating five differential wheat cultivars/lines. Based on virulence phenotypes, 63 isolates were classified as race 1, and 30 isolates were assigned to race 3. A subset of 42 isolates was selected for molecular characterization with the presence or absence of the *ToxA* and *ToxB* genes. The results showed that 36 isolates out of 42 tested by polymerase chain reaction (PCR) and Southern analysis lacked the *ToxA* and *ToxB* genes. Six isolates harboring the *ToxA* and *ToxB* genes induced necrosis and chlorosis on Glenlea and 6B365, respectively. Thirteen *ToxA* gene-deficient isolates also caused necrosis and chlorosis on Glenlea and 6B365, respectively; however, they did not fit current race classification. In contrast, the remaining 23 *ToxA* gene-deficient isolates did not cause necrosis, but induced chlorosis on 6B365, showing a disease profile for race 3. When the virulence of AR LonB2 (an isolate with unclassified race) was compared with known races 1, 3, and 5 of *P. tritici-repentis* on 20 winter wheat cultivars from Arkansas, the virulence phenotypes differed substantially. Taken together, the *ToxA* and *ToxB* gene-deficient isolates of *P. tritici-repentis* that induce necrosis and/or chlorosis may produce a novel toxin(s) on wheat.

Tan spot, which is caused by *Pyrenophora tritici-repentis*, is one of the major foliar diseases of wheat (*Triticum aestivum* L.) worldwide. The disease can reduce yield up to 50% when highly susceptible wheat cultivars are planted (23,38,40). Due to changes in cultural practices (e.g., minimum or zero tillage), the cultivation of susceptible varieties, and changes in the virulence of the pathogen, tan spot has become a serious concern for wheat producers in the United States (12).

Traditionally, pathogenic races of *P. tritici-repentis* have been characterized based on the ability of isolates to induce necrosis and/or chlorosis on five differential wheat cultivars/lines (4,5,26–28,30,31). Eight races of *P. tritici-repentis* have been identified worldwide (42). At present, races 1 through 5 exist in the United States (1,5,14,17). The ability of *P. tritici-repentis* to induce necrosis and chlorosis on wheat is correlated with the production of host-selective toxins (HSTs). *P. tritici-repentis* produces three HSTs including Ptr ToxA,

Ptr ToxB, and Ptr ToxC (10,11,13,42). Race 1 produces both Ptr ToxA and Ptr ToxC, race 3 produces Ptr ToxC, and race 5 produces Ptr ToxB (10,11,13,42). Among these, both Ptr ToxA and Ptr ToxB are proteaceous toxins (10,11,46).

Emerging evidence suggests that Ptr ToxA is internalized into the mesophyll cells and localizes to the chloroplast of sensitive wheat cultivars (34,36). Multiple motifs are required for Ptr ToxA activity on sensitive wheat (32). In addition, Ptr ToxA interacts with a chloroplast protein, ToxA-binding protein 1 (ToxABP1) (35,39). Both Ptr ToxA and Ptr ToxB can inhibit photosystem activity in the chloroplast and may lead to the production of reactive oxygen species (ROS) (33,43). Sensitivity to Ptr ToxA is controlled by a single locus, *Tsn1*, located on chromosome 5BL in wheat (6,15). The interaction between *Tsn1* (6,15) and Ptr ToxA (11) follows an inverse gene-for-gene relationship (31,44) and triggers molecular mechanisms similar to those reported previously for avirulence–resistance gene interactions (2).

Arkansas is one of the major winter wheat–producing states in the United States, where tan spot disease is a limiting factor for wheat cultivation (22). Characterization of *P. tritici-repentis* isolates from Arkansas is imperative for developing resistant wheat varieties. When a collection of *P. tritici-repentis* isolates was evaluated

on differential wheat cultivars, several necrosis inducing (nec+) isolates of *P. tritici-repentis* were identified (1,3), which lack the *ToxA* gene (11). The present study was undertaken to gain insight into the pathogenic and molecular variations of the *P. tritici-repentis* isolates from Arkansas. The objectives of this study were to (i) determine the virulence of *P. tritici-repentis* isolates on differential wheat cultivars/lines, (ii) analyze isolates for the presence or absence of the *ToxA* and *ToxB* genes using PCR and Southern analysis, and (iii) compare the virulence of AR LonB2 (an isolate of unknown race) with known races 1, 3, and 5 of *P. tritici-repentis* on the 20 winter wheat cultivars from Arkansas.

MATERIALS AND METHODS

Collection and isolation of *P. tritici-repentis* isolates. Infected leaf samples from winter wheat varieties were collected from six counties (Arkansas, Craighead, Cross, Jackson, Lonoke, and Prairie) in Arkansas in 2002. Each leaf sample was dissected into 1- to 2-cm pieces, and 30 leaf segments were placed in plastic petri dishes (9 cm diameter) containing three layers of moist Whatman no. 1 filter paper. The petri dishes were incubated in an alternating cycle of 24 h light (22°C) followed by 24 h darkness (16°C) for 96 h to induce conidiophore production and conidial development, respectively. After incubation, leaf sections were examined using a stereomicroscope, and a single conidium of the fungus was removed with a sterilized steel needle. Single spores were transferred to V8 potato dextrose agar (V8-PDA; 150 ml of V8 juice, 10 g of Difco PDA, 3 g of CaCO₃, 10 g of agar, and 850 ml of distilled water) (Difco Laboratories, Detroit, MI) plates and incubated at 21°C in darkness for 8 days. In total, 93 single-spore isolates were obtained and stored as described previously (24).

Inoculum preparation. Each isolate of *P. tritici-repentis* was revived by transferring two plugs of the fungal mycelium onto V8-PDA plates. Inoculated plates were wrapped in aluminum foil and maintained at 21°C for 5 days in an incubator. Each plate was then flooded with approximately 30 ml of sterile distilled water, and the fungal mycelium was flattened manually with a sterile glass rod. The plates were incubated in an alternating cycle of 18 to 24 h light at room tempera-

Corresponding author: T. B. Adhikari
E-mail: tika.adhikari@ndsu.edu

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ture (21 to 23°C) followed by 18 to 24 h of darkness (16°C). After profuse sporulation, sterilized distilled water (30 ml) was added to each petri dish and inoculum concentration was adjusted to 3,000 conidia/ml prior to inoculation. Two drops of Tween 20 (polyoxyethylene sorbitol ester; Sigma-Aldrich, Inc., St. Louis, MO) was added to spore suspension (100 ml) as a surfactant.

Virulence analysis. Variations in the virulence of 93 isolates of *P. tritici-repentis* were evaluated on five differential wheat cultivars/lines: Glenlea, 6B365, 6B662, Salamouni, and Katepwa (26,29,42). Wheat cultivar Glenlea harbors *Tsn1*, the gene controlling sensitivity to Ptr ToxA (races 1, 2, 7, and 8); wheat line 6B365 harbors *Tsc1*, the gene controlling sensitivity to Ptr ToxC (races 1, 3, 6, and 8); and wheat line 6B662 harbors *Tsc2*, the gene controlling sensitivity to Ptr ToxB (races 5, 6, 7, and 8) (18,30,31,42,44). Ptr ToxA induces necrosis on Glenlea, and Ptr ToxB and Ptr ToxC cause chlorosis in wheat lines 6B362 and 6B365, respectively (31). For example, symptoms induced by race 1 on wheat cultivar/line Glenlea and 6B365 are presented in Figure 1. Katepwa possesses the genes for sensitivity to Ptr ToxA and Ptr ToxB (races 1, 2, 5, 6, 7, and 8), and these HSTs cause necrosis and/or chlorosis on Katepwa, depending on the production of Ptr ToxA or Ptr ToxB, respectively (31,42). Salamouni is resistant to all known races and insensitive to all three HSTs (31,42).

Three seeds of each cultivar/line were planted in plastic cones (3.8 cm in diameter and 20 cm long) (Stuewe and Sons, Inc., OR) filled with Sunshine Mix #1 (Fison Horticulture, Vancouver, B.C.). Two-week-old seedlings were inoculated individually with each isolate to runoff using a CO₂-pressurized sprayer. Following inoculation, plants were transferred to a mist chamber to provide continuous leaf wetness. After 24 h, seedlings were moved into a growth chamber at 21/18°C (day/night) with a 16 h light/8 h dark photoperiod. The *P. tritici-repentis* race 1 (a reference isolate # Pti2) was originally collected from a wheat field in South Dakota and was included in this study because it is widely distributed (1,5,17) and produces both Ptr ToxA and Ptr ToxC

(11,42). The virulence of each isolate was assessed 8 days postinoculation (dpi) according to a disease scale of 1 to 5 as proposed previously (26,27), where 1 = small brown to black spot without tan necrosis and chlorosis; 2 = small dark brown spot with minute amounts of tan necrosis or chlorosis; 3 = small brown to black spot surrounded by a distinct ring of tan necrosis or chlorosis and not coalescing; 4 = small brown to black spot surrounded by tan necrosis or chlorosis, sometimes coalescing; and 5 = most lesions consisting of coalescing tan necrotic or chlorotic tissues.

DNA extraction. A subset of 42 isolates was randomly selected and characterized for the presence or absence of the *ToxA* and *ToxB* genes (Table 1). Conidia of each isolate were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth. Inoculated flasks were incubated at 22 ± 2°C at 100 rpm for 72 h, and mycelia were then harvested from individual flasks by filtering the inoculum through two layers of Whatman no. 1 filter paper. Mycelia of individual isolates were lyophilized and ground to a fine powder using a high-speed mixer mill (Model MM301; Retsch Inc., Newtown, PA). Genomic DNA was extracted using either DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) or a method described previously (17).

Ptr ToxA-based PCR analysis. Primers were chosen to correspond to the coding region of Ptr ToxA. The designed primers amplified a 408-bp region from the middle of the *ToxA* gene including both intron and exon regions. The primers used were SnToxA Cod1F 5'-CTACAGGAG CGGCAGGAAGC-3' and SnToxA Cod1R 5'-GCTGCATTCTCCAATTTCACG-3' (19). All PCRs were performed at the conditions using an annealing temperature of 60°C. Each reaction (10 µl) consisted of 200 ng of template DNA, 1 µl of 10× buffer, 0.5 units of *Taq* polymerase (New England BioLabs, Ipswich, MA), 4 pmol of forward and reverse primers, and 0.8 µl of 2.5 mM dNTPs. Each PCR was performed on a Dyad Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), and the cycling parameters were 40 cycles of the following: denaturation 60 s at 94°C, annealing 60 s

at 60°C, and extension 3 min at 72°C. The PCR product was visualized on a 1% agarose gel in 1× TBE (10.8 g of Tris Base, 5.5 g of boric acid, 5.8 g of EDTA, and 1,000 ml of deionized water) running buffer.

Southern analysis. The 42 isolates of *P. tritici-repentis* (Table 1) were evaluated for the presence or absence of the *ToxA* gene (11) using Southern analysis as described previously (16). Primers ToxA-*EcoRI* 5'-GAATTCATGCGTTCATCCTCGTACTT CT-3' and ToxA-*XbaI* 5'-TCTAGACTA ATTTTCTAGCTGCATTCTCCA-3', which contain *EcoRI* and *XbaI* restriction sites, respectively, were used to amplify and clone the *ToxA* coding region into a TOPO TA cloning vector as described by the manufacturer (Invitrogen Corp., Carlsbad, CA). Probe labeling, hybridization, and posthybridization washes were done as described previously (16).

Ptr ToxB-based PCR analysis. The 42 selected isolates of *P. tritici-repentis* (Table 1) were analyzed by the ToxB specific primer set (7). Each PCR product (20 µl) was separated in 2% (wt/vol) agarose gels (Sigma-Aldrich, Inc., St. Louis, MO) in 0.5× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Electrophoresis was conducted at 115 V for 90 min, and gels were stained with ethidium bromide for 15 min and photographed under UV light.

Comparison of virulence of isolate AR LonB2 with known races 1, 3, and 5 of *P. tritici-repentis* on winter wheat cultivars. Seeds of 20 Arkansas winter wheat cultivars (Table 2) were kindly supplied by Eugene A. Milus, University of Arkansas, Fayetteville and were grown in plastic cones as described above. A representative isolate AR LonB2 from Arkansas, which resembles race 3 and lacks the *ToxA* gene, was included for comparison with known race 1 (isolate # Pti2), race 3 (isolate # Ptr 331-9), and race 5 (isolate # Ptr DW7) of *P. tritici-repentis*. The isolate Ptr 331-9 was kindly provided by L. Lamari, University of Manitoba, Winnipeg, Canada. Inoculum preparation and inoculation were performed as described above. Two-week-old seedlings of each wheat cultivar were inoculated individually and assessed disease reactions 8 dpi based on a rating scale of 1 to 5 (26).

The experiments were laid out in a randomized complete block design (RCBD) with three replications in 2007. Two experiments were conducted in growth chambers, and the environmental conditions were the same in both growth chambers to represent biological replications. In the statistical analysis, the replications were regarded as a random effect, and the wheat cultivars/lines were considered as a fixed effect. Three plants of each cultivar/line were cultivated in individual cones (3.8 cm in diameter and 20 cm long) (Stuewe and Sons), and each cone was

Fig. 1. Symptoms induced by race 1 (isolate # Pti2) of *Pyrenophora tritici-repentis* on differential wheat cultivars Glenlea and 6B365. Upper panel shows tan necrosis (Nec⁺) and lower panel shows extensive chlorosis (Chl⁺).

considered as an experimental unit. In total, nine plants per experiment were rated for each isolate. Plants were considered to be resistant when the lesion types were <3.0 and susceptible when the lesion types were >3.0 as described above.

Ptr ToxA bioassay. Twenty winter wheat cultivars from Arkansas were evaluated for reactions to Ptr ToxA (Table 2). Purified Ptr ToxA was kindly provided by Steven Meindhart, NDSU, Fargo, ND. Three leaves of each wheat cultivar were infiltrated with Ptr ToxA (10 µg/ml) at the two-leaf stage with a 1-ml needleless syringe (13). The plants were placed in a growth chamber and maintained as mentioned above. Wheat cultivars Glenlea (sensitive to Ptr ToxA) and Salamouni (insensitive to Ptr ToxA) were used as checks. The infiltrated leaves were evaluated for the presence of necrosis (indicating toxin sensitivity) 72 h after infiltration.

Culture filtrate of AR LonB2 was obtained as described previously (37). Approximately 25 µl of the culture filtrate was infiltrated into the second leaf of seedlings using a 1-ml needleless syringe until water-soaking of tissue was observed. The infiltrated leaf was marked with a nontoxic permanent marker, and the plant response to the culture filtrate was recorded 4 days after infiltration. Wheat cultivars Erik (insensitive to Ptr ToxA, Ptr ToxB, and Ptr ToxC), 6B365 (sensitive to Ptr ToxC), 6B662 (sensitive to Ptr ToxB), and Katepwa (sensitive to Ptr ToxA and Ptr ToxB) were included as checks. The plants were rated as sensitive (S) or insensitive (I) based on the presence or absence of necrosis or chlorosis 4 days after infiltration as described previously (27).

RESULTS

Virulence analysis. Of the 93 isolates tested, 63 induced necrosis and chlorosis on Glenlea and 6B365, respectively, but were avirulent on 6B662 and Salamouni. The 63 isolates were assigned to race 1 (*data not shown*). Among them, 19 isolates induced necrosis on Glenlea (sensitive to Ptr ToxA), but only six isolates harbored the *ToxA* gene (Table 1). For example, disease symptoms induced by AR CrossA5 on four wheat cultivars/lines, Glenlea, 6B662, 6B665, and Salamouni, are presented in Figure 2. The 30 remaining isolates resembled race 3 as they exhibited chlorosis on 6B365; however, these isolates were avirulent on Glenlea and 6B662 (*data not shown*).

Ptr ToxA-based PCR and Southern analyses. Genotypic analysis revealed that 13 of the 19 isolates of *P. tritici-repentis*, which were designated race 1 based on phenotypic analysis, lacked the *ToxA* gene, suggesting that these isolates may not be race 1 (Table 1). Six isolates (AR ArA5, AR JacB3, AR LonA5, AR PraA1, AR PraA5, and AR PraB1) were found to har-

bor the *ToxA* gene, which is typical of race 1, and the remaining samples were genomic DNA from "race 1-like isolates" that lack the *ToxA* gene (Figs. 3 and 4).

Ptr ToxB-based PCR assay. As expected, the majority of the isolates of *P. tritici-repentis* lacked the *ToxB* gene (Fig. 5). Among the 42 isolates tested, six isolates (AR CrB10, AR CrossB3, AR JacA5, AR JacB3, AR LonA5, and AR LonB5) harbored the *ToxB* gene. None of the isolate induced chlorosis on 6B662 (sensitive to Ptr ToxB).

Virulence of isolate AR LonB2 and known races 1, 3, and 5 of *P. tritici-repentis* on winter wheat cultivars. The 20 winter wheat cultivars were susceptible to race 1 (Table 2). Nine wheat cultivars were susceptible to race 3, while all cultivars were resistant to race 5 (Table 2). The results showed that four wheat cultivars (Delta Grow 1600, Delta Grow 4500, Pat, and Pioneer 26 R15) were resistant to race 3 but were susceptible to AR LonB2 (Table 2). Similarly, four wheat cultivars (Agri Pro/Coker 9593, Agri Pro/Coker Magnolia,

Table 1. Phenotypic and genotypic analysis of *Pyrenophora tritici-repentis* isolates collected from Arkansas

Isolate ^a	Virulence assay ^b	Ptr ToxA-based PCR assay ^c	Ptr ToxA-based Southern analysis ^d	Ptr ToxB-based PCR assay ^e
AR ArA1	Nec+Chl+	—	—	—
AR ArA2	Nec—Chl+	—	—	—
AR ArA5	Nec+Chl+	+	+	—
AR ArB10	Nec—Chl+	—	—	—
AR ArB2	Nec—Chl+	—	—	—
AR CrA1	Nec+Chl+	—	—	—
AR CrA10	Nec—Chl+	—	—	—
AR CrA3	Nec—Chl+	—	—	—
AR CrA5	Nec+Chl+	—	—	—
AR CrB1	Nec+Chl+	—	—	—
AR CrB10	Nec+Chl+	—	—	+
AR CrB2	Nec—Chl+	—	—	—
AR CrB3	Nec—Chl+	—	—	—
AR CrossA1	Nec—Chl+	—	—	—
AR CrossA10	Nec+Chl+	—	—	—
AR CrossB1	Nec+Chl+	—	—	—
AR CrossB10	Nec+Chl+	—	—	—
AR CrossB2	Nec—Chl+	—	—	—
AR CrossB3	Nec—Chl+	—	—	+
AR CrossB5	Nec+Chl+	—	—	—
AR JacA1	Nec+Chl+	—	—	—
AR JacA5	Nec—Chl+	—	—	+
AR JacB10	Nec+Chl+	—	—	—
AR JacB3	Nec+Chl+	+	+	+
AR JacB4	Nec—Chl+	—	—	—
AR LonA1	Nec+Chl+	—	—	—
AR LonA10	Nec—Chl+	—	—	—
AR LonA3	Nec—Chl+	—	—	—
AR LonA5	Nec+Chl+	+	+	+
AR LonA8	Nec—Chl+	—	—	—
AR LonB1	Nec—Chl+	—	—	—
AR LonB10	Nec—Chl+	—	—	—
AR LonB2	Nec—Chl+	—	—	—
AR LonB4	Nec—Chl+	—	—	—
AR LonB5	Nec—Chl+	—	—	+
AR LonB6	Nec—Chl+	—	—	—
AR LonB9	Nec—Chl+	—	—	—
AR PraA1	Nec+Chl+	+	+	—
AR PraA3	Nec+Chl+	—	—	—
AR PraA5	Nec+Chl+	+	+	—
AR PraB1	Nec+Chl+	+	+	—
AR PraB5	Nec—Chl+	—	—	—
Race 1 (isolate # Pti2)	Nec+Chl+	+	+	—

^a Isolates originating from Arkansas State (AR) are followed by abbreviations indicating the county of origin. For example, Ar, Cr, Cross, Jac, Lon, and Pra indicate isolates originating from Arkansas, Craighead, Cross, Jackson, Lonoce, and Prairie counties, respectively.

^b Variations in the virulence of 42 isolates of *P. tritici-repentis* were based on their ability to induce necrosis and/or chlorosis on five differential wheat cultivars/lines—Glenlea, 6B365, 6B662, Salamouni, and Katepwa—8 days postinoculation (26,27,29). Abbreviations: Nec+ and Nec— represent the presence and absence of necrosis on wheat cultivar Glenlea, respectively; Chl+ refers to the presence chlorosis on wheat line 6B365.

^c *P. tritici-repentis* isolates were analyzed by polymerase chain reaction (PCR) for the presence of the *ToxA* gene as described previously (19).

^d To reconfirm the presence of the *ToxA* gene in Arkansas isolates, Southern analysis was performed as described previously (16).

^e Isolates were analyzed for the presence of the *ToxB* gene as described previously (7).

Pioneer 26 R87, and Progeny 166) were susceptible to race 3, but these cultivars were resistant to AR LonB2, indicating that this isolate could be different from race 3.

Ptr ToxA bioassay. Glenlea and Salamouni exhibited sensitive (necrosis) and insensitive (no necrosis) reactions to Ptr ToxA, respectively. None of the 20 winter wheat cultivars from Arkansas exhibited necrosis when infiltrated with Ptr ToxA, indicating that these wheat cultivars do not possess the *Tsn1* gene (Table 2).

DISCUSSION

Considerable success has been achieved in understanding wheat-*P. tritici-repentis* interactions in the last two decades. A complex structure consisting of eight races of *P. tritici-repentis* has been reported on the basis of differential responses on the wheat cultivars/lines (31,42). Molecular analysis revealed that the three HSTs produced by *P. tritici-repentis* are virulence determinants that can distinguish the fungus in terms of races (11,13,27,37). In this study, 93 isolates of *P. tritici-repentis* collected from Arkansas were analyzed for their virulence on five wheat cultivars/lines and for the presence or absence of the *ToxA* and *ToxB* genes based on PCR and Southern blot analyses. The results showed

that the six isolates produced necrotic (similar to race 1) and chlorotic (similar to races 1 and 3) symptoms on Glenlea and 6B365, respectively, but were avirulent on 6B662 and Salamouni. It appeared that these six isolates can be categorized as race 1 according to the current race classification scheme (26,27,42). Another group contained 13 isolates that induce necrosis on Glenlea but did not harbor the *ToxA* gene. Therefore, these isolates did not fit in the current race classification, suggesting that these isolates may contain different toxin compounds that can induce a similar disease phenotype (Ptr

ToxA-type necrosis) on sensitive wheat cultivar Glenlea.

A differential wheat cultivar Katepwa has been used to identify race 5 of *P. tritici-repentis* in Canada (30). Based on disease reactions, race 5 was virulent on Katepwa, but not on 6B365 (30). In contrast, race 3 isolates from Canada were avirulent on Katepwa (30). We show that the 30 isolates resembling race 3 were capable of inducing chlorosis on Katepwa, indicating that these isolates possess an additional virulence factor. This finding is similar to that of previous studies in the wheat-*P. tritici-repentis* interactions

Fig. 2. Reactions of isolate AR CrossA5 (unclassified race) of *Pyrenophora tritici-repentis* from Arkansas on wheat cultivars/lines. AR CrossA5 induced necrosis on Glenlea and chlorosis on 6B365, respectively, but was avirulent (no disease symptoms) on 6B662 and Salamouni.

Table 2. Reactions of 20 winter wheat cultivars from Arkansas to isolate AR LonB2 (unclassified race) and three known races, 1, 3, and 5, of *Pyrenophora tritici-repentis*

Winter wheat cultivars ^a	Race 1 (isolate # Pti2)	Race 3 (isolate # Ptr 331-9)	Race 5 (isolate # DW7)	AR LonB2 (CF) ^b	AR LonB2	Purified Ptr ToxA ^c
Agri Pro/Coker 9593	4.6 (S)	3.9 (S)	1.0 (R)	I	1.2 (R)	I
Agri Pro/Coker Beretta	4.0 (S)	1.6 (R)	1.3 (R)	I	1.5 (R)	I
Agri Pro/Coker Magnolia	4.6 (S)	3.5 (S)	1.1 (R)	I	2.2 (R)	I
AGS 2000	4.3 (S)	1.6 (R)	1.1 (R)	I	1.4 (R)	I
AGS 2050	4.3 (S)	3.5 (S)	2.5 (R)	I	3.9 (S)	I
Armor 5110	4.2 (S)	3.1 (S)	1.0 (R)	I	4.2 (S)	I
Croplan Genetics 554W	4.1 (S)	1.8 (R)	1.1 (R)	I	1.9 (R)	I
Croplan Genetics 8302	4.1 (S)	1.3 (R)	1.2 (R)	I	2.9 (R)	I
Delta Grow 1600	4.2 (S)	1.3 (R)	1.1 (R)	I	3.3 (S)	I
Delta Grow 4500	4.6 (S)	1.7 (R)	1.0 (R)	I	4.1 (S)	I
Delta King 7710	4.3 (S)	3.9 (S)	1.7 (R)	I	4.3 (S)	I
Dixie 989	4.8 (S)	3.4 (S)	1.1 (R)	I	3.6 (S)	I
Hornbeck 3266	4.9 (S)	4.2 (S)	2.1 (R)	I	4.2 (S)	I
Pat	3.9 (S)	1.0 (R)	1.0 (R)	I	4.1 (S)	I
Pioneer 26 R15	4.8 (S)	1.6 (R)	1.1 (R)	I	3.4 (S)	I
Pioneer 26 R22	4.8 (S)	1.6 (R)	1.2 (R)	I	2.2 (R)	I
Pioneer 26 R87	4.9 (S)	3.7 (S)	1.5 (R)	I	1.2 (R)	I
Progeny 166	4.8 (S)	3.6 (S)	1.1 (R)	I	2.0 (S)	I
Terral 8558	4.7 (S)	1.8 (R)	1.0 (R)	I	1.5 (R)	I
Terral LA 481	4.7 (S)	1.2 (R)	1.0 (R)	I	1.9 (R)	I
6B365	5.0 (S)	4.9 (S)	1.1 (R)	I	4.8 (S)	I
Erik (resistant check)	1.0 (R)	1.0 (R)	1.0 (R)	I	1.1 (R)	I
Katepwa	4.5 (S)	N/A	4.5 (S)	I	3.9 (S)	S
6B662	N/A	1.5 (R)	4.5 (S)	I	2.0 (R)	I

^a Plants were rated on a 1 to 5 lesion-type scale (26). Plants with ratings <3.0 were classified as resistant (R), and those with ratings >3.0 were classified as susceptible (S). Wheat cultivars/lines Erik, 6B662, and 6B365 were used for race confirmation and inoculation validation. Erik was used as a resistant check, and Katepwa and 6B365 were used as susceptible checks for race 1 confirmation and inoculation validation; Erik and 6B365 were used for race 3 verifications and inoculation validation; 6B662 was used for race 5 verification and inoculation validation. S = susceptible, R = resistant, and N/A = not available.

^b Culture filtrate (CF) (25 µl) from isolate AR LonB2 was introduced into the second leaf of wheat seedlings until water-soaking was observed. Four days after infiltration, plants were rated as sensitive (S) or insensitive (I) based on presence and absence of necrosis or chlorosis at the infiltration site, respectively.

^c Three leaves of each wheat cultivar were infiltrated with purified Ptr ToxA (10 µg/ml) using a 1-ml needleless syringe at the two-leaf stage as described previously (13). Infiltrated plants were placed in a growth chamber. The leaves were assessed for their reaction based on the presence (toxin sensitive = S) or absence (toxin insensitive = I) of necrosis 72 h after infiltration.

(21,42), which also predicted the production of additional toxins based on the reactions of certain wheat genotypes. Further studies are necessary to investigate the

presence of additional toxin in *P. tritici-repentis* race 3 from Arkansas.

A detailed molecular analysis for detecting HSTs is necessary to better understand

race structure and dynamics of variation in *P. tritici-repentis* populations. Although Ptr ToxB is associated with chlorosis on 6B662 and Katepwa, and Ptr ToxC is asso-

Fig. 3. Agarose gels showing the presence or absence of the *ToxA* gene in 42 isolates of *Pyrenophora tritici-repentis* from Arkansas detected by Ptr ToxA-based polymerase chain reaction (PCR) analysis. Lane 1: 200-bp DNA ladder.

Fig. 4. Detection of the *ToxA* gene in 42 isolates of *Pyrenophora tritici-repentis* from Arkansas by ToxA-based Southern analysis. Lane 1: Lambda DNA.

ciated with chlorosis on 6B365, both Ptr ToxA and Ptr ToxB have predominantly been found in separate isolates (30,31,44). We used a ToxA-based PCR assay to determine if the *ToxA* gene was present in *P. tritici-repentis* isolates. The Ptr ToxA-based PCR analysis confirmed by Southern analysis suggested that these isolates designated as race 1 based on the virulence phenotypes did not correspond to race 1 using a molecular genotyping. The 36 isolates out of 42 characterized in this study lacked the *ToxA* gene. Our results suggest that these isolates possess a novel toxin(s) that enables them to induce necrotic symptoms similar to those of Ptr ToxA.

Three chlorosis-inducing (nec+chl+) isolates (AR CrossB3, AR JacA5, and AR LonB5) resembling race 3 harbor the *ToxB* gene (Table 1). Another three necrosis- and chlorosis-inducing (nec+chl+) isolates (AR CrB10, AR JacB3, and AR LonA5) also carry the *ToxB* gene. However, the majority (36 out of 42 isolates) were capable of inducing chlorosis on wheat line 6B365 but lacked the *ToxB* gene. The *P. tritici-repentis* races 5, 6, 7, and 8 harbor the *ToxB* gene, which is known to produce Ptr ToxB (31,42). Recent findings show that *ToxB*-like sequences have been found in

races 3 and 4 of the fungus, which do not possess Ptr ToxB activity (41), as well as in other members of ascomycetes that do not infect wheat (8). Additionally, the form of Ptr ToxB from avirulent race 4 isolates, which lack chlorosis-inducing activity, has been found in Canada (25). We did not perform sequence analysis of the isolates tested in this study. Thus, it is uncertain whether the chlorosis-inducing activity was due to the form of the *ToxB*-like sequences present or reaction of the host to infection.

Race 3 isolates of *P. tritici-repentis* have been reported from different continents (1,17,42). The development of race 3 specific primers would facilitate the identification of chlorosis-inducing isolates of *P. tritici-repentis* (7). One of the limitations of the present study is lack of a screening procedure for the *ToxC* gene in *P. tritici-repentis* race 3 isolates from Arkansas. Although Ptr ToxC can induce chlorosis in wheat line 6B365, this toxin is highly unstable (S. W. Meinhardt, *personal communication*).

In Arkansas, cultivation of wheat began in the early 1960s, and approximately 1.014 million acres were planted with winter wheat from 1993 to 2003 (9). However, tan spot disease was only first re-

ported on wheat in Arkansas in 1989 (22). The 20 winter wheat cultivars from Arkansas were susceptible to race 1, but these were insensitive to Ptr ToxA. It appears that *Tsn1* is not present in most wheat cultivars grown in Arkansas. Although the mechanisms of the evolution of a new race(s) in the fungal population from Arkansas are unknown, an effort to identify a new pathogenic race(s) and to characterize a novel toxin(s) in *P. tritici-repentis* isolates is in progress. This will advance our knowledge on the intricacies of this pathosystem.

In conclusion, the Arkansas isolates infect wheat cultivars differentially, and may provide an excellent opportunity to explore the relationship between *P. tritici-repentis* and wheat reported previously in this biological system as well as in *Stagonospora nodorum* (19,20), which follows an inverse gene-for-gene model. Comparison of virulence of races 1, 3, and 5 of *P. tritici-repentis* with the Arkansas isolate on wheat cultivars showed that an additional virulence factor(s) other than Ptr ToxA and Ptr ToxC is present in AR LonB2. Thus, wheat cultivars such as Delta Grow 1600, Delta Grow 4500, and Pat (Table 2) may serve as good candidates and will be of great benefit for characterizing virulence

Fig. 5. Agarose gels showing the presence or absence of the *ToxB* gene in 42 isolates of *Pyrenophora tritici-repentis* from Arkansas generated by Ptr ToxB-based polymerase chain reaction (PCR) analysis. Lane 1: 100-bp DNA ladder.

factors in the Arkansas isolates. Additionally, these novel isolates will be useful for testing resistance to *P. tritici-repentis* in wheat breeding programs.

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