Pyrenophora tritici-repentis Race 4 Isolates Cause Disease on Tetraploid Wheat

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ABSTRACT

The ascomycete fungus *Pyrenophora tritici-repentis* is the causal agent of tan spot of wheat. The disease can occur on both common wheat (*Triticum aestivum*) and durum wheat (*T. turgidum* ssp. *durum*) and has potential to cause significant yield and quality losses. The fungal pathogen is known to produce necrotrophic effectors (NEs) that act as important virulence factors. Based on the NE production and virulence on a set of four differentials, *P. tritici-repentis* isolates have been classified into eight races. Race 4 produces no known NEs and is avirulent on the differentials. From a fungal collection in North Dakota, we identified several isolates that were classified as race 4. These isolates caused no or little disease on all common wheat lines including the differentials;

however, they were virulent on some durum cultivars and tetraploid wheat accessions. Using two segregating tetraploid wheat populations and quantitative trait locus mapping, we identified several genomic regions significantly associated with disease caused by two of these isolates, some of which have not been previously reported. This is the first report that race 4 is virulent on tetraploid wheat, likely utilizing unidentified NEs. Our findings further highlight the insufficiency of the current race classification system for *P. tritici-repentis*.

Keywords: genetics and resistance, host selective toxins, inverse gene-for-gene model, mycology, pathogenicity test, QTL mapping

Tan spot is caused by the necrotrophic fungal pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.). This disease was first reported in the United States in the 1940s (Barrus 1942), but it was rarely observed until the 1970s when no-tillage practices were widely adopted (Hosford 1982; reviewed in Faris et al. 2013; Rees and Platz 1992). The typical symptoms on susceptible cultivars include tan-colored necrotic lesions, which are often surrounded by chlorotic halos or extensive chlorosis. These symptoms are associated with fungal-produced necrotrophic effectors (NEs), previously known as host-selective toxins (HSTs) (reviewed in Faris et al. 2013). In the last several decades, the disease has become one of the economically important foliar diseases in all major wheat growing regions of the world (reviewed in Faris et al. 2013). The fungal infection damages leaf tissues reducing photosynthesis activity, which can result in significant yield losses. Yield losses of nearly 50% have been reported in Australia (Rees et al. 1982). The disease can also cause red smudge on wheat kernels, particularly in durum wheat, which can lead to downgrading of wheat grains (Fernandez et al. 1996).

Outbreaks of tan spot were thought to be attributed by the adoption of conservation agriculture practices to reduce erosion and maintain soil fertility. Crop rotation, residue management, and fungicide application can be integrated to manage tan spot of wheat. However, cultural practices and fungicide applications are not

always effective. For example, it has been documented that *P. tritici-repentis* isolates have developed resistance to commonly used fungicides (Murray and Brennan 2009). The most cost effective and environmentally friendly way is to use resistant cultivars (Faris et al. 2013; Friskop and Liu 2016).

The wheat–*P. tritici-repentis* pathosystem follows an inverse gene-for-gene model where NEs produced by the pathogen interact with the products of the host sensitivity genes to induce necrosis or chlorosis leading to disease. Therefore, these NEs act as important virulence factors. To date, three NEs have been identified from *P. tritici-repentis*, namely Ptr ToxA, Ptr ToxB, Ptr ToxC, and the wheat genes interacting with them are *Tsn1*, *Tsc2*, and *Tsc1*, respectively (reviewed in Ciuffetti et al. 2010; Faris et al. 2013). Ptr ToxA is a small protein (~13.2 kDa) that induces necrosis, and it is encoded by the single copy gene *ToxA* (Balance et al. 1996; Ciuffetti et al. 1997; Manning et al. 2013). ToxA is also produced by two other wheat fungal pathogens, *Parastagonospora nodorum* and *Bipolaris sorokiniana*, to cause disease on wheat upon recognition by the product of the *Tsn1* gene (Friesen et al. 2006, 2018; Liu et al. 2006; McDonald et al. 2018; Navathe et al. 2020). It was shown that a genomic segment containing the *ToxA* gene was transferred from one fungal pathogen to another through horizontal gene transfer events (Friesen et al. 2006; McDonald et al. 2018). Ptr ToxB is also a small protein, and, unlike Ptr ToxA, it induces chlorosis, and has a smaller molecular size (6.61 kDa). The fungal gene encoding Ptr ToxB, *ToxB*, has been cloned and was shown to be present in multiple copies (Martinez et al. 2001, 2004; Strelkov et al. 2006). The *ToxB* gene is also present in *P. bromi*, a fungus causing leaf disease on grasses (Andrie et al. 2008). Some races of *P. tritici-repentis* carry a nonfunctional form of *ToxB*, designated *toxh* (Amaike et al. 2008; see below). Ptr ToxC is a chlorosis-inducing NE and was initially characterized as a nonionic, polar, low molecular weight secondary metabolite (Effertz et al. 2002). The chemical structure of Ptr ToxC and fungal gene responsible for its production remains unknown.

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The current classification system defines eight races for *P. tritici-repentis*, which is largely based on their ability to produce individual or a combination of the three NEs. This correlates with their virulence on a set of four common wheat lines including Salamouni (universally resistant), Glenlea (sensitive to Ptr ToxA), 6B662 (sensitive to Ptr ToxB), and 6B365 (sensitive to Ptr ToxC) (Lamari et al. 2003). Two durum lines, Coulter (sensitive to Ptr ToxA) and 4B1149 (insensitive to all NEs), are sometimes included in the differential set (Lamari et al. 1995), but they do not increase the power to describe additional races. All virulent races produce one known NE or more and are virulent on the differential lines carrying corresponding sensitivity gene. In contrast, race 4 produces no known NE and is avirulent to all the differentials. Using this classification system, the *P. tritici-repentis* populations from different geographic locations have been characterized (Abdullah et al. 2017a; Aboukhaddour et al. 2013; Bertagnolli et al. 2019; Gamba et al. 2012; Kamel et al. 2019; Sarova et al. 2005). Races 1 and 2 were found to be predominant in North and South America, and Australia, and relatively complicated race structures were found in the Middle East, North Africa, and Caucasus regions (Kamel et al. 2019). In addition, there were isolates identified in several studies that do not conform to the current race classification system (Ali and Franc 2002; Ali et al. 2010; Benslimane 2018; Kamel et al. 2019).

Race 4 was first described as pathotype 4 (nec- chl-), which did not produce any necrosis and chlorosis symptom on wheat differentials (Lamari and Bernier 1989b; Lamari et al. 1991, 1995). However, a cytological investigation showed that the isolates of this race can penetrate wheat epidermal cells and reach mesophyll cells (Lamari et al. 1991). This race has been shown to be rare among many *P. tritici-repentis* collections from wheat (Ali and Franc 2003; Benslimane et al. 2011; Kamel et al. 2019; Lamari and Bernier 1989b; Lamari et al. 1998; Sarova et al. 2005). However, collections from noncereal grasses had a large proportion of race 4 isolates (Ali and Franc 2003). Although race 4 does not produce any known NE and is avirulent to the differentials, it carries *toxb*, which is 86% similar to *ToxB* and can be transcribed at a very low level (Amaike et al. 2008; Martinez et al. 2004).

A collection of *P. tritici-repentis* isolates was obtained from wheat fields in North Dakota to characterize race structure in the state. Race typing on differential lines and molecular characterization has shown that a few isolates are likely race 4 (Z. Liu, unpublished data). Those isolates caused disease on some tetraploid wheat lines in our preliminary evaluations. The objectives of this study were to confirm their race 4 identity, determine their virulence on a set of common wheat and durum cultivars, and map genomic regions conferring resistance/susceptibility to two of these race 4 isolates.

MATERIALS AND METHODS

Fungal isolates. Five isolates from previous field surveys were initially characterized as race 4 based on the phenotypes they exhibited on tan spot differential lines (Z. Liu, unpublished data).

Those isolates were designated as Ls13-14, Ls13-78, Ls13-86, Ls13-192, and Ls13-198. The first three were collected in the North Dakota counties Mountrail, Kidder, and Wells, respectively, and the last two were collected in Barnes County. Those isolates were subjected to molecular characterization, pathogenicity tests, and genetic mapping of host factors conditioning resistance or susceptibility. Three isolates with known race identity including Pti2 (race 1), 86-124 (race 2), and DW5 (race 5) were used as references in the experiments.

Molecular characterization. The five isolates were first checked for their species identity by sequencing the internal transcribed spacer (ITS) regions. Genomic DNA was extracted according to the protocol described by Shjerve et al. (2014). The PCR amplifications of ITS regions were done using the universal primers ITS4 and ITS5 (Table 1) (White et al. 1990). The PCR amplifications were performed with 10 ng of DNA template in a total volume of 20 µl containing 1× buffer, 200 µM dNTPs, 1.5 mM MgCl₂, 0.5 µM of each forward and reverse primer, and 2 U of Bullseye Taq DNA polymerase (MIDSCI, St. Louis, MO). PCRs were performed with the followed cycling conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 1 min. A final elongation step was performed at 72°C for 5 min. PCR products were separated on 1% agarose gels in 1 × TAE buffer (0.04 M Tris-acetate, pH 8.0, and 0.001 M EDTA) and purified with PureLink PCR Quick PCR Purification Kit (Invitrogen) following the supplier's recommendation. The purified products were sequenced with the primers IT4 and ITS5 at the facility of Eurofins Genomics USA Inc. (Louisville, KY). The obtained sequences were compared with each other and blasted against the NCBI nonredundant database using BLASTn.

After these isolates were confirmed to be *P. tritici-repentis*, they were tested for presence of the *ToxA* and *ToxB* genes with published primer pairs (Table 1). *Mat1-1-1* was also included as a control for DNA quality and quantity (Ameen et al. 2017). In the PCR amplification, 86-124 (race 2, carrying *ToxA*) and DW5 (race 5, carrying *ToxB*) were used as positive controls for the *ToxA* and *ToxB* genes, respectively. The obtained PCR products were purified and sequenced as described above. Upon learning that these isolates contain *toxb*, we downloaded the sequence of a genomic region spanning *toxb* from GenBank (accession AY083456.2) (Martinez et al. 2004) and designed a new primer pair (toxF/toxBR) (Table 1) to amplify the full-length *toxb* open reading frame (ORF) from these isolates. The *toxb* sequences were aligned with each other as well as the reference *toxb* sequence using MultAlin program (<http://multalin.toulouse.inra.fr/multalin/>).

Plant materials and disease evaluations. The current tan spot differential lines including Salamouni, Glenlea, 6B365, and 6B662 were initially used for race typing. Pathogenicity tests of these isolates were then conducted on a set of thirty wheat lines, the majority of which were hard red spring (HRS) wheat and durum wheat cultivars currently planted in North Dakota (Table 2). Disease evaluations were carried out in the greenhouse and growth chambers under controlled conditions. Seeds of these lines were planted in Super cell cones (Stuewe and Sons, Inc., Corvallis, OR)

TABLE 1. List of primers used in this study

Primers	Sequences (from 5' to 3')	Purpose	Reference
PtrPLP2	CAGAACAAAGGCAGGACTGTGAGC	Amplify the <i>MAT1-1-1</i>	Lepoint et al. (2010)
PtrPLP4	ATGCGCTCAGCAAGGAAGGTCG		
TA51F	GCGTCTATCCTCGTACTTC	Amplify the <i>ToxA</i>	Andrie et al. (2008)
TA52R	GCAATCTCCAATTTTCACG		
TB71F	GCTACTTGCTGTGGCTATC	Amplify the <i>ToxB</i>	Andrie et al. (2008)
TB6R	ACGTCCTCCACTTTGCACACTCTC		
toxF	TGTTAATCTTATACTAATCCCTTG	Amplify the <i>toxb</i>	This study
toxBR	TCTTGTAGCGCTAAACTCT		
ITS4	TCCTCCGCTTATTGATATGC	Amplify the ITS	White et al. (1990)
ITS5	GGAAGTAAAAGTCGTAACAAGG		

with three seeds per cone. The four differential lines mentioned above were also included in the evaluations. Plants were grown in Sunshine SB100 potting mix (Sun Gro Horticulture, Bellevue, WA) with a half teaspoon of the fertilizer Osmocote Plus 15-9-12 (Scotts Sierra Horticultural Product Company, Maysville, OH) applied to each cone after planting. The cones were placed onto RL98 racks. The border cones of each rack were planted with the highly susceptible winter wheat cultivar Jerry to reduce the edge effects (Liu et al. 2015). Pathogenicity tests were conducted for four isolates (Ls13-14, Ls13-86, Ls13-192, and Ls13-198) along with Pit2, DW5, and 86-124 used as controls. Ls13-78 did not produce enough spores for pathogenicity test. For this experiment, three biological replications were carried out with a randomized complete block design (RCBD).

To identify genomic regions associated with resistance/susceptibility to tan spot caused by the race 4 isolates, we conducted disease evaluations of two isolates (Ls13-14 and Ls13-192) on two tetraploid populations. The first population was derived from the cross between Ben (sensitive to Ptr ToxA) and PI 41025 and referred as BP025, which consisted of 200 recombinant inbred lines (RILs) (Zhang et al. 2014). Ben is a North Dakota hard amber durum variety released in 1998 (Elias and Miller 1998), and PI 41025 is a cultivated emmer wheat (*T. turgidum* ssp. *dicoccum*) accession collected near Samara, Russia. The second population, referred to as RP336, was derived from a cross between the durum line Rusty (sensitive to Ptr ToxB) and the tetraploid wheat *T. turgidum* ssp. *turgidum* accession PI 387336, which consisted of 186 RILs (Liu et al. 2020a). In this experiment, disease evaluations included all the

RILs and their parental lines from each population as well as the four differential lines. Three biological replications were included with an RCBD arrangement. The planting and growing plants followed the same procedures as described above.

The fungal culturing, inoculum preparation and inoculation methods followed standard protocols described in Liu et al. (2015). Briefly, the fungus was grown on V8 potato dextran agar medium for 5 days under dark followed by mycelium flattening and a light-dark incubation cycle. Conidia were washed with sterilized distilled water from the culture plates to make spore suspension. The spore concentration was adjusted to approximately 3,000 spores/ml and two drops per 100 ml of the surfactant reagent Tween 20 were added before inoculation. Plants at the two- to three-leaf stage were inoculated using an air-pressured spray gun. After inoculation, plants were kept in a mist chamber with 100% humidity for 24 h. Then, the plants were transferred to a growth chamber with a 12-h photoperiod at 21°C. Disease reactions were scored at 7 days postinoculation using a 1 to 5 rating scale described by Lamari and Bernier (1989a). We added 0 to the scale when no obvious reaction was observed. Disease scores lower than 2.0 were considered resistant.

Statistical analysis. In pathogenicity tests, the disease mean difference between durum and common wheat groups was analyzed using the Wilcoxon test in PROC NON-PARAMETRIC in SAS 9.4 Software (SAS Institute, Cary, NC, 2016). After the disease data were obtained for the two populations, normality of the disease data for each isolate/replication was evaluated using the Shapiro-Wilk test in PROC UNIVARIATE in SAS 9.4 Software (SAS Institute,

TABLE 2. Reaction of wheat genotypes to tan spot caused by different *Pyrenophora tritici-repentis* race/isolate

Wheat type	Genotypes	Agent or origin ^a	Year released	Race 4 ^b				Race 5 ^b	Race 1 ^b	Race 2 ^b
				Ls13-14	Ls13-192	Ls13-86	Ls13-198	DW5	Pti2	86-124
Common wheat	Barlow	ND	2009	0.3	0.3	0.8	0.0	3.8	4.5	2.0
Common wheat	Brennan	AgriPro	2009	0.3	0.4	0.5	0.5	1.5	3.0	2.0
Common wheat	Glenn	ND	2005	0.3	0.9	0.5	0.0	4.0	3.0	2.5
Common wheat	Howard	ND	2000	0.3	0.7	0.3	0.0	3.5	4.0	3.0
Common wheat	Jenna	AgriPro	2009	0.2	0.4	0.0	0.0	1.0	2.5	1.5
Common wheat	Kelby	AgriPro	2006	0.2	0.8	0.5	0.0	2.5	2.5	2.0
Common wheat	Mott	ND	2007	0.8	1.3	0.8	1.0	3.0	3.5	3.5
Common wheat	Prosper	ND	2011	0.5	0.6	0.3	0.0	4.0	4.0	3.0
Common wheat	RB07	MN	2007	0.2	0.5	0.3	0.0	2.0	2.5	1.5
Common wheat	Rollag	MN	2011	0.0	0.5	0.8	0.0	4.5	2.8	3.0
Common wheat	Select	SD	2010	0.2	0.9	0.8	0.3	4.3	4.5	2.5
Common wheat	Steele-ND	ND	2004	0.5	1.0	0.5	0.3	4.3	3.5	3.5
Common wheat	SY Soren	Syngenta/AgriPro	2011	0.2	0.6	0.5	0.5	2.5	1.8	1.0
Common wheat	SY Tyra	Syngenta/AgriPro	2011	0.0	0.7	0.3	0.0	1.8	3.5	1.0
Common wheat	Velva	ND	2011	0.2	0.8	0.0	0.0	3.3	2.3	1.0
Common wheat	Chinese spring	China	–	0.3	0.3	0.5	0.0	1.8	2.0	5.0
Common wheat	Vantage	WestBred	2007	0.2	1.0	1.3	0.3	2.0	3.5	2.0
Common wheat	Briggs	SD	2002	0.2	0.6	0.5	0.0	2.0	3.5	3.0
Means for common wheat				0.3	0.7	0.5	0.2	2.9	3.2	2.4
Durum wheat	Alkabo	ND	2005	0.5	1.5	1.0	1.8	2.5	1.8	2.0
Durum wheat	Ben	ND	1996	3.8	3.4	3.3	2.8	4.5	3.0	4.0
Durum wheat	Carpio	ND	2012	2.5	2.2	0.5	2.0	3.5	2.8	2.5
Durum wheat	Dilse	ND	2002	3.5	3.3	3.3	3.0	3.8	2.5	3.5
Durum wheat	Divide	ND	1994	3.0	3.1	2.3	2.8	4.5	2.0	3.5
Durum wheat	Grenora	ND	2006	3.2	3.2	2.5	2.8	4.0	3.5	3.0
Durum wheat	Lebsock	ND	1999	3.3	3.3	2.8	3.0	4.5	2.0	2.5
Durum wheat	Langdon	ND	1956	1.7	2.4	0.8	1.8	4.5	4.5	4.0
Durum wheat	Mountrail	ND	1998	2.5	3.0	1.5	2.0	4.5	1.5	3.0
Durum wheat	Pierce	ND	2001	3.2	3.1	2.5	2.8	4.5	2.0	4.0
Durum wheat	Rusty	ND	2004	1.5	1.7	1.0	0.0	4.5	1.5	2.5
Durum wheat	Tioga	ND	2010	3.3	2.6	2.5	3.0	4.0	2.0	2.5
Emmer wheat	PI 41025	Russia	–	1.2	0.5	–	–	–	–	–
Poulard wheat	PI 387336	Ethiopia	–	3.3	3.2	–	–	–	–	–
Means for tetraploid wheat				2.6	2.6	2	2.3	4.1	2.4	3.1
Mean difference between common wheat and durum groups $P > t ^c$				<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0163	0.001

^a Private companies or public breeding programs that developed the cultivars: MN = University of Minnesota; ND = North Dakota State University; and SD = South Dakota State University, or the place the genotype came from.

^b Disease was scored using 1 to 5 scale, where 1 is resistant and 5 is susceptible. Average of three replicates were given as the final score.

^c One way analysis of variance was used to analyze the difference in disease means of common wheat and durum wheat. Difference is significant at $P > |t| < 0.05$.

2016). If the data fit a normal distribution, it was tested for homogeneity using Bartlett's χ^2 test (Snedecor and Cochran 1989). If the data did not fit a normal distribution, Levene's test was used to test the homogeneity among different replicates (Levene 1960). The data from homogeneous replications were combined and the means were used for quantitative trait locus (QTL) analysis.

QTL analysis. QTL analysis employed the genetic linkage maps of the two populations that have been published previously (Liu et al. 2020a, b). BP025 was genotyped several times through different methods including simple sequence repeats (SSRs), Illumina iSelect 9K SNPs array, and genotyping-by-sequencing (GBS). The genetic linkage map for this population contained a total of 4,883 markers (96 SSRs, 878 9k, and 3,909 GBS) with a total length of 4,749.6 cM covering all 14 chromosomes. The genetic map of the RP336 population contained 2,894 GBS markers and had a total length of 2,858 cM covering all 14 chromosomes. QTL mapping was conducted using QGene 4.4.0 (Joehanes and Nelson 2008). Composite interval mapping (CIM) performed with forward

cofactors selection was used to identify the genomic region associated with tan spot reaction and to quantify the disease variations explained by the QTL (R^2). A permutation test with 1,000 iterations resulted in a logarithm of the odds (LOD) threshold of 4.2 for an experiment-wise significance level of 0.05. To find out whether the QTLs detected in this study possibly represent known genes or genetic loci, the physical positions and the markers linked were compared. The physical locations of markers were obtained using marker sequences as queries in BLASTn searches of the IWGSC Chinese Spring reference genome (v1.0).

RESULTS

Species and race confirmation. Inoculation of all four isolates (Ls13-14, Ls13-86, Ls13-192, and Ls13-198) on four differentials (Salamouni, 6B662, 6B365, and Glenlea) produced no or little disease. Most reactions were pin-sized black dots either alone or surrounded by small amounts of chlorosis (Fig. 1). Almost no reaction could be seen on the resistant line Salamouni. However, the three control isolates Pti2 (race 1), DW5 (race 5), and 86-124 (race 2) caused a large amount of necrosis or chlorosis on their differential lines. These results indicated that the four Ls13 isolates belonged to race 4.

A partial ITS sequence (436 bp) was obtained for all five isolates after removal of low-quality base readings. The ITS sequences were identical for all five isolates. BLASTn searches showed that these sequences were 100% identical to the published ITS sequences of *P. tritici-repentis* strains 90-2T (AM887495) (Lepoint et al. 2010), 90-2 (EF452479) (Andrie et al. 2008), and SY3-122 (AY739819) (Friesen et al. 2005), all of which were characterized as *P. tritici-repentis* race 4. The partial ITS sequences for all five isolates have been deposited as GenBank accession numbers MN855580 to MN855584.

All isolates lacked the *ToxA* gene, but they produced weak amplification with a band size of approximate 250 bp using the *tox*b primer set TB71F/TB6R (Fig. 2). The fragments were sequenced and confirmed to be the *tox*b sequence. Using a new pair of primers (*tox*bF and *tox*bR) (Table 1), we amplified the full-length fragment of the *tox*b gene from all five isolates. Sequence analysis indicated that the *tox*b ORF (267 bp) was identical among all five isolates) and

Fig. 1. Reaction of wheat differential lines to tan spot caused by *Pyrenophora tritici-repentis* race 4 isolates and other known races. **A**, Ls13-192; **B**, Ls13-86; **C**, Ls13-14; **D**, Ls13-198; **E**, Pti2 (race 1); **F**, 86-124 (race 2); and **G**, DW5 (race 5).

Fig. 2. PCR testing of the presence of the *ToxA* and *ToxB* genes in race 4 isolates. *ToxA* was multiplexed with *MAT1-1*. Isolates 86-124, DW5, and H₂O were included in the PCR as controls. Arrows indicate size of DNA markers and the individual bands.

have been deposited in NCBI GenBank (MN864562 to MN864566). BLASTn analysis using the full-length sequence indicated that the *toxB* sequence of these five isolates was 100% identical to that of race 4 isolate SD20 (AY083456.2) (Martinez et al. 2004), and 99% identical to that of race 4 isolate 90-2 (AF483832) (Strelkov and Lamari 2003). The sequence was 87% similar to the *ToxB* gene sequence from the race 5 isolate DW7 (AY425484) (Martinez et al. 2004). Therefore, these results further confirmed that these isolates are race 4.

Pathogenicity tests. The results of pathogenicity test of four isolates on thirty wheat lines are shown in Table 2. All HRS wheat cultivars had disease means less than 1.0 for all four isolates indicating nonpathogenic or avirulent reactions of these race 4 isolates on common wheat genotypes. The average disease rating was 0.26 for Ls13-14, 0.68 for Ls13-192, 0.49 for Ls13-86, and 0.15 for Ls13-198. Most HRS wheat cultivars developed no visible symptom or black pinpoint lesions on the leaves, e.g., Jenna, Steele-ND, and Vantage, whereas other cultivars such as Mott and Prosper developed small and restricted necrotic lesions (Fig. 3). For the reference isolates Pti2, DW5, or 86-124, all HRS cultivars except Jenna and SY Soren showed highly susceptible or susceptible reactions.

For tetraploid wheat, the disease means ranged from 0.5 (e.g., Ls13-14 on Alkabo) to 3.8 (Ls13-14 on Ben), indicating variable reactions to these race 4 isolates (Table 2). Some of the reactions were characterized as being susceptible because their disease means were greater than 3.0. The average disease ratings were 2.66 for Ls13-14, 2.73 for Ls13-192, 1.98 for Ls13-86, and 2.29 for Ls13-198. The disease reactions were observed as large necrotic lesions surrounded by yellow margin on highly susceptible lines (Fig. 3). This indicates that these race 4 isolates can cause disease on some tetraploid genotypes. For reaction to the reference isolates, most of durum cultivars were highly susceptible to DW5 (race 5), but less susceptible to race 1 and 2 isolates.

Comparison of disease ratings between tetraploid and hexaploid wheat groups found they differ significantly difference in reaction to race 4 isolates as well as race 1, 2, and 5 isolates (Table 2).

QTL identification. Based on the results from pathogenicity tests, Ben and PI 41025 as well as Rusty and PI 387336 differed greatly in reaction to the four race 4 isolates we identified (Table 2, Fig. 3). Therefore, the BP025 and RP336 populations were used to identify genomic regions associated with reaction to tan spot caused by the race 4 isolates Ls13-14 and Ls13-192 on each population. Normality tests rejected a normal distribution of the disease reaction to both isolates ($P < 0.05$). Homogeneity analysis indicated that the variance among the replicates for each isolate were not significant ($P = 0.12$ to 0.44). Therefore, the means of three biological replicates for each isolate were used in subsequent data analysis and QTL identification. Both populations segregated for reaction to the two isolates, exhibiting a range from highly resistant to highly susceptible reactions, and no obvious transgressive segregation was observed (Fig. 4). The majority of RILs in BP population had disease scores lower than 3.0 while the RP336 population skewed to susceptible reaction.

Six QTLs were identified in BP025 with three for each isolate (Table 3). The QTLs were distributed on chromosomes 1A (designated as *BP-ls192-1A* and *BP-ls14-1A*), 4B (*BP-ls192-4B* and *BP-ls14-4B*) and 5A (*BP-ls192-5A* and *BP-ls14-5A*) (Fig. 5, Table 3). The resistance alleles for all the QTLs were contributed by PI 41025. The LOD values for these QTLs ranged from 4.8 to 13.0 and they explained from 11 to 26% of the variation in disease. The 5A QTL for two isolates (*BP-ls192-5A* and *BP-ls14-5A*) colocalized to the same physical interval (660 to 672 Mb), whereas the 1A and 4B QTLs for the two isolates were located to different intervals. The 5A and 4B QTLs had slightly higher effect on disease compared with the 1A QTL (Table 3).

For the RP336 population, two QTLs (*RP-ls192-5A* and *RP-ls192-4B*) were identified for Ls13-192 and one (*RP-ls14-5A*) for Ls13-14 (Table 3, Fig. 5). *RP-ls192-5A* and *RP-ls14-5A* were

located at the same physical interval and explained a large portion of disease variations. This 5A QTL is most likely the same as the one identified in the BP025 population according to the physical locations of flanking markers. However, the 4B QTL was in a different physical interval compared with the ones identified in the BP025 population.

DISCUSSION

P. tritici-repentis isolates are classified into eight races according to their virulence on a set of wheat lines, including four common wheat and two durum lines, with race 4 being avirulent to all those lines (Aboukhaddour et al. 2013; Lamari and Strelkov 2010). So far, many studies have reported the identification of race 4 isolates from wheat and alternative hosts (Abdullah et al. 2017a, b; Ali and Franci 2003; Benslimane et al. 2011; Friesen et al. 2005; Kamel et al. 2019; Lamari et al. 1995, 1998, 2005; Singh et al. 2007). Little attention has been paid to race 4 isolates because they were assumed to be avirulent to all wheat lines. It has been an intriguing question as to why race 4 exists in nature without causing disease. In this work, we confirmed the race 4 identity of five isolates collected from North Dakota using pathogenicity tests and molecular characterization. Although these isolates were avirulent to all common wheat lines tested, they caused disease on some durum cultivars and other tetraploid wheat genotypes. Using two segregating tetraploid

Fig. 3. Reaction of representative wheat lines to fungal inoculation with race 4 isolates. **A**, Common wheat lines inoculated with Ls13-192. **B**, Durum wheat lines inoculated with Ls13-192. **C**, The parental lines for two tetraploid population inoculated with Ls13-192 and Ls13-14. Disease scores for all wheat genotypes to different *Pyrenophora tritici-repentis* race/isolates are listed in Table 2.

populations, we further showed that there are several genomic regions in wheat that govern reaction to these race 4 isolates. Benslimane (2018) reported five isolates that caused no disease on common wheat, but on durum lines. However, these isolates were not considered to be race 4 because they carry both *ToxA* and *ToxB* and no *toxb*. The study speculated that *Ptr ToxA* and *Ptr ToxB* were not expressed for some reasons. Therefore, our study was the first to show that race 4 isolates cause disease on durum cultivars and other tetraploids. This might provide an explanation for why race 4 can be consistently isolated from infected wheat leaves and is present in nature. Our finding also suggests that durum breeding programs should include race 4 isolates in their disease screenings. Because common wheat and durum have shown many differences in reaction to different *P. tritici-repentis* races, it might be necessary to take different approaches in the breeding of tan spot resistance for two different types of wheats.

The five race 4 isolates used in this study were obtained from a collection of 352 isolates obtained from North Dakota wheat fields (Z. Liu, unpublished data). Unfortunately, we did not record the wheat type or cultivar they were collected from; thus, it remains unknown if they were collected from durum wheat. Previous studies have shown that race 4 can be isolated at low percentages (4 to 20%) from bread wheat (Abdullah et al. 2017b; Ali and Francl 2003; Lamari et al. 1991, 1995, 1998) and at high percentages (40 to 98%) from alternative hosts such as noncereal grasses and rye (Abdullah et al. 2017a; Ali and Francl 2003). There were also reports of race 4 being occasionally isolated from durum wheat (Benslimane et al. 2011; Kamel et al. 2019). It would be important to examine if the previously reported race 4 isolates from other geographic regions and hosts are virulent on durum. Further research is needed to

systematically investigate the race structure of the fungal populations collected from durum and how prevalent race 4 is in durum wheat production areas. All such information will be important for durum wheat breeding programs. In addition, we observed that our race 4 isolates caused small necrotic lesions on some common wheat cultivars, and thus it is important to test these race 4 isolates on a wider range of common wheat cultivars.

Although *P. tritici-repentis* has eight races described, the genetic and evolutionary relationship among them remains unclear. According to Hosford (1982) and Maraite (1997), the fungus was first isolated from quack grass (*Agropyrum repens*, originally called *Triticum repens*) in 1903 and then from wheat in 1928. Before the 1940s, the fungus was described as a saprophyte or minor pathogen on wheat, but after that it became a highly virulent pathogen on wheat, probably through acquisition of the NE gene *ToxA* (Friesen et al. 2006). Our speculation is that the fungus first appeared on wheat as race 4 and later evolved into other virulent races by acquiring different virulence genes. Population genetic studies have revealed a clear genetic separation of race 4 from all other virulent isolates (Aboukhaddour et al. 2011; Aung 2001). This genetic separation was also supported by a recent whole genome phylogenetic analysis (Moolhuijzen et al. 2018). Considering that wheat evolved from tetraploid to hexaploid levels, it is possible that race 4 gained virulence first on tetraploid wheat by obtaining the *ToxB*, *ToxC*, and other unknown virulence genes, and then on hexaploid wheat by acquiring the *ToxA* gene. This speculation can be supported by several lines of evidence: (i) highly similarly homologs of *ToxB* were identified from the related grass pathogen *P. bromi* and large percentage of race 4 could be isolated from the grasses (Ali and Francl 2003; Andrie et al. 2008; Andrie and

Fig. 4. Histogram of disease reactions to tan spot caused by the race 4 isolates in BP025 and RP336 populations. The parental reactions are indicated by arrows.

TABLE 3. Quantitative trait locus (QTL) associated with reaction to tan spot caused by Ls13-192 and Ls13-14 from composite interval mapping analysis in the BP025 and RP336 population

Population	Isolate	QTL	Genetic interval (cM)	Physical interval (Mbp)	LOD ^a	R ² (%) ^b	Flanking markers
BP025	Ls13-192	<i>BP-ls192-1A</i>	92–96	51–56	4.8	0.11	<i>S1A_56880271-S1A_51221934</i>
		<i>BP-ls192-4B</i>	58–60	53–106	13	0.26	<i>S4B_53863164-S4B_106853468</i>
		<i>BP-ls192-5A</i>	408–410	668–672	7.6	0.17	<i>S5A_668189084-S5A_672505022</i>
	Ls13-14	<i>BP-ls14-1A</i>	118–120	168–299	5.8	0.13	<i>S1A_168577107-S1A_299657544</i>
		<i>BP-ls14-4B</i>	110–114	535–563	6.2	0.14	<i>S4B_535050204-S4B_563205252</i>
		<i>BP-ls14-5A</i>	404–408	666–670	11.6	0.25	<i>S5A_666689644-S5A_670630031</i>
RP336	Ls13-192	<i>RP-ls192-4B</i>	60–64	621–639	6.2	0.15	<i>4B_621667742-4B_639997885</i>
		<i>RP-ls192-5A</i>	236–238	666–674	19.8	0.41	<i>5A_666503690-5A_674124822</i>
	Ls13-14	<i>RP-ls14-5A</i>	236–238	666–674	17.2	0.39	<i>5A_666503690-5A_674124822</i>

^a Permutation test with 1,000 iterations yielded a logarithm of the odds (LOD) value of 4.2 and it was used as the cut-off to identify significant QTL.

^b R² value indicates the amount of phenotypic variation explained by the individual QTL.

Ciuffetti 2011); (ii) *P. tritici-repentis* races 3 and 5, which produce the individual NEs Ptr ToxC and Ptr ToxB, respectively, were mostly isolated from durum wheat (Ali and Franc 2003; Kamel et al. 2019; Lamari and Bernier 1989b; Lamari et al. 2005); and (iii) The Ptr ToxB–*Tsc2* and Ptr ToxC–*Tsc1* interactions play important roles in disease on both tetraploid wheat and common wheat whereas Ptr ToxA–*Tsn1* interaction is only important for common wheat, i.e., the *ToxA* gene acts as a virulence factor on hexaploid but not tetraploid wheat (Faris et al. 2012, 2020; Galagedara et al. 2020; Liu et al. 2020a, b; Viridi et al. 2016). There are numerous subspecies of tetraploid wheat, which have longer and more complex evolutionary and domestication histories than hexaploid wheat. Determining the pathogenicity or virulence of race 4 isolates on different tetraploid subspecies might provide a better picture of the evolution of *P. tritici-repentis* races. In addition, the D genome progenitor *Aegilops tauschii* may play a role in *P. tritici-repentis* evolution because susceptibility has been reported in some accessions (Alam and Gustafson 1988; Friesen et al. 2008).

As mentioned, the current race classification system for *P. tritici-repentis* is largely based on the ability of the isolate to produce one or more of the three NEs, and the wheat lines with sensitivity to each of the three NEs serve as the differential set. Therefore, this system only allows the description of eight races (2³) (Lamari et al. 2003). The limitation of the current race classification system was

demonstrated by the fact either that the existing races produced additional virulence factors or that some isolates with no production of all three NEs caused disease on one of the differential lines (Ali et al. 2010; Friesen et al. 2003; Gamba and Lamari 1998; Guo et al. 2018; Kamel et al. 2019; Lamari et al. 1995). Our research further indicates there is a need to revise the current classification system by adding durum and other tetraploid wheat, which has been suggested by previous studies (Lamari and Bernier 1991; Singh et al. 2007). Here, we showed that at least some race 4 isolates can cause disease on tetraploid wheat; therefore, the susceptible tetraploid wheat lines, such as Ben and PI 387336, are suggested to be included in the differential set. However, adding more differential lines would significantly increase the number of races, which would make disease system difficult to study and follow. The expansion of the race classification system should rely on further dissection of the genetics of host–pathogen interactions in both hexaploid and tetraploid wheats. The development of differential lines with individual susceptibility factors would be useful to describe and encompass all possible races.

Although race 4 is nonpathogenic, most race 4 isolates have been reported to carry *toxB*, which is 87% homologous to *ToxB* (Martinez et al. 2004; Strelkov et al. 2006). *ToxB* encodes Ptr ToxB, a chlorosis-inducing protein, and is present and highly expressed in isolates of races 5, 6, 7, and 8. The copy number, gene sequence and

Fig. 5. The genomic regions identified by quantitative trait locus (QTL) associated the development of tan spot disease caused by the race 4 isolates Ls13-192 and Ls13-14. A physical scale in mega base pairs for wheat chromosomes is shown to the left. The solid black box on each chromosome indicates the region significantly associated with tan spot on different population and caused by different isolate. Markers on the left of each chromosome define the physical region of the QTL. The QTLs are listed to the right of the chromosomes, with different colors for population and isolate combinations, including BP025 with Ls13-192 (red), BP025 with Ls13-14 (orange), RP336 with Ls13-192 (blue), and RP336 with Ls13-14 (purple). The LOD and *R*² values for each QTL are presented in Table 3.

role in virulence for *ToxB* has been extensively investigated (Ciuffetti et al. 2010). However, there were only two *tox**b* ORF sequences available in NCBI GenBank, SD20 and 90-2, which were collected in the United States (South Dakota) and Canada, respectively (Martinez et al. 2004; Strelkov and Lamari 2003). The *tox**b* gene was also shown to be expressed in mycelium tissues and conidia of 90-2 (Amaike et al. 2008; Martinez et al. 2004; Strelkov et al. 2006). The race 4 isolates we collected in North Dakota all carry identical *tox**b* sequences, which were identical to that of SD20 but slightly different (99% homology) from 90-2. We did not measure the expression of *tox**b* for the ND isolates, but it would be an interesting to determine if *tox**b* functions in virulence on these tetraploid wheat genotypes.

To verify virulence of the race 4 isolates, we inoculated two of these isolates (Ls13-192 and Ls13-14) onto two segregating tetraploid wheat populations (BP025 and RP336) and identified wheat genomic regions conferring resistance or susceptibility to them using QTL mapping. In total, we identified six genomic regions distributed on chromosomes 1A, 4B, and 5A (Table 3, Fig. 5). Among them, the genomic region on chromosome 5A was identified for both isolates and populations indicating a common QTL in tetraploid wheat associated with reaction to race 4 isolates. Several regions on chromosome 5A have been previously identified to harbor tan spot resistance or susceptibility QTL on both hexaploid and tetraploid wheat with virulent races (Chu et al. 2008, 2010; Hu et al. 2019; Kariyawasam et al. 2016; Liu et al. 2020a; Virdi et al. 2016). Our 5A QTL was physically located within 666.5 to 674.1 Mbp, which likely corresponds to the region for *QTs.fcu-5A.2*, *RP336_86-124_5A.2*, *RP696_86-124_5A.2*, and *RP336_86-124ΔToxA_5A* (Chu et al. 2010; Liu et al. 2020a). These QTLs were identified with races 1 and 2 or their *ToxA* knockout strains. This suggests that race 4 isolates might carry the same virulence factor as other virulent races that interact with this 5A QTL to cause disease.

Three genomic regions on 4B were identified with one (*BP-ls192-4B*) on the short arm and the other two (*BP-ls14-4B* and *RP-ls192-4B*) on the long arm, and they were specific to an individual population or isolate. No tan spot-associated QTL has previously been reported on 4B in hexaploid wheat, but Virdi et al. (2016) identified a QTL (*QTs.fcu-4B*) on 4BS in a tetraploid wheat associated with disease caused by both L13-35 (race 2) and DW5 (race 5). This QTL might be in proximity of the QTL *BP-ls192-4B* identified in this research. However, the two QTLs on 4BL seem to be novel. Two closely linked regions on chromosome 1A were identified in the BP025 population, but not in RP336 suggesting this QTL might be host specific. Many QTLs have been identified on 1AS for tan spot resistance or susceptibility, but they are mainly associated with disease attributed to the Ptr ToxC–*Tsc1* interaction (Faris et al. 2013; Kariyawasam et al. 2016; Liu et al. 2017). However, race 4 does not produce Ptr ToxC and the physical location for *Tsc1* tends to be more distal end of chromosome 1A (Liu et al. 2020b). Thus, these QTLs identified here unlikely correspond to the *Tsc1* locus and are also novel. The identification of novel QTL suggests that these race 4 isolates carry unidentified and unique virulence factor(s) to cause disease on tetraploid wheat genotypes.

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