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Inheritance of Virulence and Linkages of Virulence Genes in an Ethiopian Isolate of the Wheat Stripe Rust Pathogen (*Puccinia striiformis* f. sp. *tritici*) Determined Through Sexual Recombination on *Berberis holstii*

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Abstract

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is one of the most devastating wheat diseases in Ethiopia. To study virulence genetics of the pathogen, 117 progeny isolates were produced through sexual reproduction of an Ethiopian isolate of the stripe rust pathogen on *Berberis holstii* plants under controlled conditions. The parental and progeny isolates were characterized by phenotyping on wheat lines carrying single *Yr* genes for resistance and genotyped using 10 polymorphic simple sequence repeated (SSR) markers. The progeny isolates were classified into 37 virulence phenotypes and 75 multilocus genotypes. The parental isolate and progeny isolates were all avirulent to resistance genes *Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *YrTr1*, *YrSP*, and *Yr76* but virulent to *Yr1* and *Yr2*, indicating that the parental isolate was homozygous avirulent or homozygous virulent at these loci. The progeny isolates segregated for virulence

to 12 *Yr* genes. Virulence phenotypes to *Yr6*, *Yr28*, *Yr43*, and *Yr44* were controlled by a single dominant gene; those to *Yr7*, *Yr9*, *Yr17*, *Yr27*, *Yr25*, *Yr31*, and *YrExp2* were each controlled by two dominant genes; and the virulence phenotype to *Yr8* was controlled by two complementary dominant genes. A linkage map was constructed with seven SSR markers, and 16 virulence loci corresponding to 11 *Yr* resistance genes were mapped with some loci linked to each other. These results are useful in understanding host-pathogen interactions and selecting resistance genes to develop wheat cultivars with highly effective resistance to stripe rust.

Keywords: cereals and grains, epidemiology, field crops, fungi, pathogen diversity

Wheat is the fourth most important staple food crop in Ethiopia, grown on 1.7 million hectares with an estimated annual production of 4.6 million metric tons (Central Statistics Agency 2016). Wheat is a key strategic crop for improving food security in the country (Negassa et al. 2013). However, wheat production in Ethiopia is prone to diverse biotic and abiotic stresses, among which wheat rust diseases are most important.

Stripe rust, caused by *Puccinia striiformis* Westend. f. sp. *tritici* Erikss. (referred to hereafter as *P. striiformis* f. sp. *tritici*), is a substantial threat to wheat production in Ethiopia (Wellings 2011). The disease is also globally important because of the fast development of new races that overcome resistance in wheat cultivars (Chen 2005; Wellings 2011). For example, resistance gene *Yr9* was overcome by the outbreak of new virulence races of *P. striiformis* f. sp. *tritici* in the late 1980s, which resulted in continuous epidemics of stripe rust in Ethiopia and many other countries (Chen et al. 2014;

Wellings 2011). Yield loss caused by stripe rust in Ethiopia ranged from 40 to 100% depending on the susceptibility of cultivars and environmental conditions (Badebo et al. 2001). The stripe rust outbreak in 2010 damaged more than 600,000 hectares of wheat fields in Ethiopia and led to the cost of more than \$3.2 million spent on fungicide applications to reduce yield losses. Two popular bread wheat cultivars possessing *Yr27*, Kubsu and Galama, were withdrawn from production as a result of the emergence of new races virulent to the resistance gene (Abeyo et al. 2014). Ethiopia and other neighboring countries in the east African highlands are at high risk of stripe rust attributable to the two-crop system of wheat and aggressive *P. striiformis* f. sp. *tritici* races (Wellings 2011).

Stripe rust is managed primarily by growing resistant cultivars and applying fungicides (Chen 2014). However, genes for race-specific resistance often lose effectiveness as a result of virulence change in the pathogen population, and fungicide application adds additional cost to wheat production and potentially damages the environment (Chen 2013, 2014). Hence, the best approach to control this disease is developing wheat cultivars with effective and durable resistance (Chen 2013). Changes in pathogen populations can be caused by mutation, introduction, genetic drift, sexual recombination, somatic hybridization, and host selection (Little and Manners 1967; Park and Wellings 2012). In most cases, populations of sexually produced pathogens usually exhibit a high degree of genotype diversity, and a recombining pathogen can combine virulence genes as rapidly as breeders can recombine resistance genes in cultivars (McDonald and Linde 2002).

Alternate hosts of *P. striiformis* f. sp. *tritici* were unknown until the recent detection of aecia of closely related fungus *P. pseudostriiformis* (syn. *P. striiformis* f. sp. *poae*) on barberry plants (Jin et al. 2010). This finding led to the discovery of several *Berberis* and *Mahonia* species as alternate hosts for *P. striiformis* f. sp. *tritici* after

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they were tested with germinating *P. striiformis* f. sp. *tritici* teliospores under controlled conditions (Jin et al. 2010; Wang and Chen 2013; Zhao et al. 2013). The discovery of *Berberis* spp. as alternate hosts of *P. striiformis* f. sp. *tritici* offers a great opportunity to study the genetics of the pathogen (Rodríguez-Algaba et al. 2014; Tian et al. 2016; Wang et al. 2012, 2018; Yuan et al. 2018). Wang et al. (2012) produced 19 isolates through sexual fertilization of a U.S. *P. striiformis* f. sp. *tritici* isolate on *B. vulgaris* under controlled conditions and observed segregation of avirulence/virulence corresponding to 14 *Yr* genes. Rodríguez-Algaba et al. (2014) obtained 16 isolates through self-fertilization of an European isolate on the same *Berberis* species and identified 11 multilocus genotypes (MLGs) using 14 simple sequence repeated (SSR) markers. In China, Tian et al. (2016) produced 118 isolates from a single Chinese *P. striiformis* f. sp. *tritici* isolate through selfing it on *B. shensiana* and identified 24 virulence phenotypes (VPs) by testing the isolates on 24 *Yr* single-gene lines of wheat. The 118 isolates were characterized as 82 MLGs using 13 SSR markers. They developed a linkage group consisting of 10 SSRs and eight avirulence/virulence loci. Similarly, Wang et al. (2018) generated 127 progeny isolates from a different Chinese isolate, CYR32, and identified 65 MLGs. They constructed two linkage groups consisting of 10 SSR markers and 10 virulence/avirulence loci. In the United States, Yuan et al. (2018) produced 119 progeny isolates developed by self-crossing *P. striiformis* f. sp. *tritici* isolate 08-220 (race PSTv-11) on *B. vulgaris* and develop a genetic map consisting of six linkage groups using a large number of genotype-by-sequencing (GBS) single nucleotide polymorphism (SNP) markers. These studies reported both dominance and recessiveness of avirulence or virulence.

Different races of *P. striiformis* f. sp. *tritici* have been reported in Ethiopia and neighboring countries in eastern Africa (Badebo et al. 2008; Sharma-Poudyal et al. 2013; Wan et al. 2017). Barberry plants commonly grow adjacent to wheat fields in Ethiopia and other eastern African countries, but whether they play a role in stripe rust is not clear. *B. holstii* is endemic to the highlands of Ethiopia (Edward et al. 2000) and was shown to be susceptible to *P. striiformis* f. sp. *tritici* under controlled conditions (Jin et al. 2010). The objectives of the present study were to determine inheritance of virulence and identify virulence genes and molecular markers by phenotyping and genotyping a sexual population produced from isolate EK3-12, representing the predominant *P. striiformis* f. sp. *tritici* race in Ethiopia, through self-fertilization on *B. holstii* plants. Information on genetic variation, including virulence/avirulence phenotypic changes, of a *P. striiformis* f. sp. *tritici* population produced through sexual fertilization is useful to provide a genetic basis for selecting resistance genes for developing wheat cultivars with effective and durable resistance.

Materials and Methods

Urediniospore and teliospore production of the parental isolate. The parental isolate EK3-12 was obtained from a stripe rust sample collected from a wheat field in southeastern Ethiopia in 2015. EK3-12 was selected from more than 100 isolates in an Ethiopian *P. striiformis* f. sp. *tritici* collection for this study because it produced a VP similar to the most predominant race of *P. striiformis* f. sp. *tritici* in Ethiopia in a recent study (Wan et al. 2017) and showed a high capability to produce abundant telia under greenhouse conditions. Because of its high capability of producing teliospores, this isolate is ideal for producing a sexual population to study the inheritance of its avirulence/virulence if it has heterozygous loci for the phenotypic reactions observed on *Yr* single-gene lines.

Urediniospores were recovered, purified, and multiplied in the State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection at Northwest A&F University in Yangling, Shaanxi, China, following a previously described procedure (Wan et al. 2004). Briefly, seedlings of susceptible wheat variety Mingxian 169 were grown in pots 10 cm in diameter and 10 cm in height in a rust-free greenhouse. After the first leaves were fully expanded, seedlings were inoculated with fresh urediniospores mixed with talc at a

1:20 ratio. Inoculated seedlings in a pot were covered with a plastic cylinder to prevent contamination, incubated in a dew chamber for 24 h at 10°C without light, and then transferred to a growth chamber with 16 h of light at 16°C and 8 h of darkness at 13°C. Urediniospores were harvested 18 to 22 days after inoculation.

Teliospores were produced by inoculating Mingxian 169 plants at the flag-leaf stage with urediniospores under the same conditions as described above. When abundant uredinia were produced approximately 14 days after inoculation, the temperature in the growth chamber was increased to 25°C during the day and 16°C at night to enhance telial production (Zheng 2011). Telia-bearing leaves were collected at approximately 1 month after inoculation, dried at room temperature for 2 to 3 days, put in paper bags, and kept in a desiccator at 4°C for approximately a week before use for inoculating barberry plants.

Inoculation of barberry leaves with basidiospores. Seeds of *B. holstii* collected from Northern Showa, Ethiopia (9.61892°N, 39.48554°E, and 2,886 m above sea level), in 2016 were planted in pots and the plants were grown in a greenhouse at Northwest A&F University. The plants with young leaves were used for inoculation. Teliospore germination and barberry inoculation were conducted following the procedures as described by Zhao et al. (2013). Before inoculation, wheat leaves bearing *P. striiformis* f. sp. *tritici* telia were washed under tap water, soaked in distilled water at 10°C in darkness for 24 h, rinsed with distilled water, and kept on 2% water agar in Petri dishes in a growth chamber at 10 to 12°C for 24 h. Teliospore germination was checked periodically under a light microscope. Immediately after teliospore germination, the Petri dish was inverted and put on top of the plastic cylinder surrounding the barberry seedlings grown in a pot. The inoculated barberry plants were kept in a dew chamber for 3 to 4 days with 100% relative humidity and a daily light period of 12 h at 16°C and 12 h of darkness at 10°C. Plants were moved to a growth chamber with a daily photocycle of 16 h of light at 16°C and 8 h of darkness at 13°C. At approximately 7 to 10 days after inoculation, pycnia were observed on the adaxial surface of the barberry leaves, producing nectar on pycnial ostioles. Nectar containing pycniospores from a pycnium was transferred to another pycnium with a paint brush soaked with sterile distilled water for fertilization. To maximize the diversity in the progeny population, nectar from one pycnium was used to fertilize only one single pycnium, and only the individual barberry leaves bearing one pycnium were used for receiving pycniospores. The plants bearing fertilized pycnia were individually isolated with plastic cylinders to prevent secondary fertilization. When aecia appeared on the basial surfaces of the barberry leaves approximately 7 to 10 days after fertilization, the plastic cylinders were removed to reduce moisture and prevent aeciospore release.

Development of progeny isolates. When an aecium matured, aecial cups reached 2 to 3 mm in length. The cups were gently cut, placed on a glass slide, and gently pressed to release aeciospores. Under a stereo microscope, an individual aeciospore was picked with a fine glass fiber and placed on a leaf surface of Mingxian 169 at the one-leaf stage with one aeciospore per leaf. Three to five leaves in a single pot were inoculated with individual aeciospores from a single aecium. The inoculated wheat plants were kept in a dew chamber at 10°C for 24 h in darkness and were then moved to a growth chamber at a diurnal cycle of 16 h of light at 16°C and 8 h of darkness at 13°C. When chlorotic lesions appeared approximately 7 to 9 days after inoculation, infected plants in a pot were transplanted separately into different pots, and each pot was covered with a plastic cylinder to prevent cross-contamination. Approximately 18 to 22 days after aeciospore inoculation, urediniospores produced on a single leaf were collected with a glass tube and stored in a desiccator at 4°C for further use. To maximize the diversity of the progeny population, only one single aeciospore-derived uredinial isolate from each aecium was included in the mapping population. Urediniospores were multiplied on Mingxian 169 seedlings to produce a large enough quantity for the phenotyping and genotyping experiments. Progeny isolates produced by self-fertilization were considered as an F₂ generation, and heterokaryotic urediniospores were considered as diploid.

Virulence testing. The parental isolate and progeny isolates were phenotyped for their VP on 22 *Yr* single-gene wheat lines used to differentiate *P. striiformis* f. sp. *tritici* races (Wan and Chen 2014) (Supplementary Table S1). Mingxian 169 was used as a susceptible check in the virulence phenotyping test. For each wheat genotype, five to seven seeds were planted in a pot 10 cm in diameter and 10 cm in height filled with cow dung, compost, and soil (1:2:1 by volume), and the plants were grown in a rust-free greenhouse. The inoculation and growth conditions were the same as described above. Infection type (IT) data were recorded 18 to 20 days after inoculation based on a 0 to 9 scale described by Line and Qayoum (1992). ITs 0 to 6 were considered avirulent and 7 to 9 were virulent (Wan and Chen 2014). If an isolate produced different ITs on different plants of a genotype, the isolate was tested on the genotype again. If both tests had different ITs on different plants, the IT on the majority of plants was used.

DNA extraction. Genomic DNA of parental and progeny isolates was extracted from urediniospores following the protocol described by Aljanabi and Martinez (1997) with modifications. Approximately 40 mg of fresh urediniospores was placed in a 2-ml centrifuge tube with steel beads (4 mm in diameter) and ground for 10 min to a fine powder in a swing mill (TissueLyser II; QIAGEN, Hilden, Germany), and 500 μ l of preheated cetyltrimethylammonium-bromide extraction buffer (0.4 M of NaCl, 10 mM of Tris-HCl, pH 8, and 2 mM of EDTA, pH 8) was added. The solution in the tube was blended gently, incubated at 65°C in a water bath for 1 h, and gently shaken every 10 min; 750 μ l of phenol chloroform-isoamyl alcohol (25:24:1 by volume, pH 8) was added and centrifuged at 12,000 rpm for 10 min at 4°C. The upper part of the aqueous solution was transferred to a new tube, 300 μ l of chloroform was added, and the mixture was centrifuged at 12,000 rpm for 10 min. The upper part was transferred to a new tube; 800 μ l of precooled isopropanol was added, and the sample was kept at -20°C for 24 h and centrifuged at 12,000 rpm at 4°C for 20 min. After the supernatant was removed, the DNA pellet was washed with 75% ethanol and then with 100% ethanol. The DNA pellet was air-dried and dissolved in 50 μ l of 1 $\times$ TE buffer (10 mM of Tris-HCl and 1 M of EDTA, pH 8.0). The solution was mixed with 1.0 ml of 20 μ l/ml RNase solution and incubated at 37°C for 30 min to digest RNA. The DNA concentration was measured using an ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and quality was checked using 0.8% agarose gel electrophoresis. The gel was stained in an ethidium bromide solution (0.5 μ g/ml) for 15 min and then visualized under an Alpha Imager System (Alpha Innotech, San Jose, CA). DNA samples were diluted to 50 ng/ μ l with 1 $\times$ TE buffer and kept at 4°C for later use.

Genotyping with SSR markers. A total of 111 SSR markers (Supplementary Table S2) (Bahri et al. 2009; Bailey et al. 2013; Cheng et al. 2012; Enjalbert et al. 2002; Luo et al. 2015; Tian et al. 2016) were screened to identify markers that were usable for genotyping the progeny population. Primers of the markers were synthesized and labeled with fluorescence at the 5' end (Shanghai Sangon Bio-tech Company, Shanghai, China). The PCR reaction consisted of 12.5 μ l of 2 $\times$ Taq Master Mix (0.05 U/ μ l of Taq DNA polymerase, 4 mM of MgCl₂, and 0.4 mM of dNTPs), 1.0 μ l of each primer (10 mM), 2.0 μ l of DNA, and 8.5 μ l of ddH₂O. The amplification conditions were as follows: a predenaturation step at 94°C for 4 min; 10 cycles of denaturation at 94°C for 45 s, annealing at 64°C for 45 s (the temperature decreased by 0.7°C for every cycle), and extension at 72°C for 45 s; 25 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 75°C for 45 s; and a final extension step at 72°C for 10 min. After PCR amplification, 5 μ l of 6 $\times$ formamide loading buffer was added to each product and denatured at 95°C for 5 min. The PCR products were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Pittsburgh, PA). Lengths of the SSR amplicons were determined using Gene Marker (Holland and Parson 2011). Heterozygosity of the SSR loci was determined using Arlequin software version 3.11 (Schneider et al. 2000).

After screening with the parental isolates, 10 SSR markers showing codominant bands in the parental isolate were used to test the progeny isolates.

Data analyses. The virulence data were analyzed using VAT 1.0 software (Kosman and Leonard 2007). χ^2 tests were conducted to determine the goodness of fit of segregation for the avirulence and virulence categories for both single-locus and two-loci models. For the marker data, only the 1:2:1 ratio for the homozygous allele A/heterozygous/homozygous allele B for a single locus was tested for each marker. *P* values > 0.05 were used to accept the tested inheritance model.

A linkage map was constructed with SSR markers fitting the 1:2:1 ratio using the JoinMap 4.1 program (Van Ooijen 2006). Because of the limited number of SSR markers, a relatively low value of the logarithm of odds (LOD = 2) was used to connect markers. The three genotypes (AA, AB, and BB) were used for each marker locus. Because the progeny population was developed from a single isolate, the highest association between alleles at different marker loci was used to connect alleles into haplotypes. Quantitative trait locus (QTL) mapping was conducted with IT data of the progeny isolates on the 0 to 9 scale using IciMapping software (Zhang et al. 2010). A QTL with an LOD value > 3 was considered significant.

Results

Virulence characterization of the sexual population. A total of 117 progeny isolates were produced through sexual reproduction of the parent isolate EK3-12 on barberry plants. IT data on the parental and progeny isolates produced on all 22 wheat lines possessing single but different *Yr* genes are provided in Supplementary Table S3. The parental isolate and all progeny isolates were avirulent to *Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *YrTr1*, *YrSP*, and *Yr76*, indicating that the parental isolate was homozygous at the avirulence loci corresponding to these *Yr* genes. In contrast, the parental isolate and all progeny isolates were homozygous virulent to *Yr1* and *Yr2*, indicating that the parental isolate was homozygous at the virulence loci corresponding to these two *Yr* genes. The parental isolate was virulent to *Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr27*, *Yr43*, *YrExp2*, *Yr25*, and *Yr31*, but the progeny isolates segregated for virulence/avirulence on these wheat lines, showing that the parental isolate was heterozygous for recessive avirulence or dominant virulence to these resistance genes. Similarly but in a different direction, the parental isolate was avirulent to *Yr8*, *Yr28*, and *Yr44*, whereas the progeny isolates segregated, indicating that the parental isolate was heterozygous at these avirulence loci. Segregations of virulence/avirulence were observed on 12 *Yr* single-gene lines.

Based on the virulence/avirulence phenotypes to the 12 *Yr* genes, the progeny isolates were classified into 37 VPs (Table 1). VP1 had 28 isolates virulent to all 12 *Yr* genes, whereas all other VPs had different avirulence/virulence patterns on the 12 *Yr* genes. Of the 37 VPs, 14 VPs, with a total of 63 isolates, had broader virulence spectra than the parental isolate; one VP (VP2 with 19 isolates) had the same virulence spectrum as the parental isolate; and 22 VPs, with a total of 35 isolates, had narrower virulence spectra than the parental isolate.

Twelve VPs had two to eight isolates and the remaining 23 VPs each had only one isolate. The deviation from a normal distribution in the number of isolates for the VPs indicated linkage relationships between some of the virulence/avirulence loci.

Segregation of progeny isolates. Inheritance of virulence loci that segregated among the progeny isolates was determined (Table 2). The segregations of VPs to *Yr6* and *Yr28* and avirulence phenotypes to *Yr43* and *Yr44* fit for a 1:3 avirulent/virulent ratio, indicating that these virulent or avirulent phenotypes were controlled by a single locus and the virulence alleles were dominant or the avirulence alleles were recessive. Therefore, the virulence genes corresponding to resistance genes *Yr6*, *Yr28*, *Yr43*, and *Yr44* were designated as *VYr6*, *VYr28*, *VYr43*, and *VYr44*, respectively. The segregation of avirulence to *Yr8* fit a 7:9 ratio (*P* = 0.40) for avirulence to virulence but also marginally fit a 9:7 ratio (*P* = 0.04) (Table 2). As the parental isolate was avirulent (IT 4), the 9:7 ratio was more reasonable, indicating that two dominant complementary genes are controlling for the

partial avirulence phenotype. Thus, the genes for the VPS were named *VYr8-1* and *VYr8-2*. Segregations of the VPs to the remaining seven *Yr* gene lines (*Yr7*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, *Yr31*, and *YrExp2*) best fit a 1:15 ratio for avirulence to virulence, indicating two independent dominant genes for virulence. These virulence genes were named

VYr7-1 and *VYr7-2*, *VYr9-1* and *VYr9-2*, *VYr17-1* and *Yr17-2*, *VYr25-1* and *VYr25-2*, *VYr27-1* and *VYr27-2*, *VYr31-1* and *VYr31-2*, and *YrExp2-1* and *YrExp2-2* corresponding to the resistance genes, respectively.

Linkage analysis and gene mapping. Of the 111 SSR markers screened for suitable markers, only 10 produced two allelic

Table 1. Virulence phenotypes (VPs) of the parental isolate EK3-12 and progeny isolates of *Puccinia striiformis* f. sp. *tritici* on wheat *Yr* single-gene lines

VP	Isolate	Virulent (V) or avirulent (A) reaction on wheat <i>Yr</i> single-gene lines											
		<i>Yr6</i>	<i>Yr7</i>	<i>Yr8</i>	<i>Yr9</i>	<i>Yr17</i>	<i>Yr25</i>	<i>Yr27</i>	<i>Yr28</i>	<i>Yr31</i>	<i>Yr43</i>	<i>Yr44</i>	<i>YrExp2</i>
VP-P ^a	EK3-12	V	V	A	V	V	V	V	A	V	V	A	V
1	28	V	V	V	V	V	V	V	V	V	V	V	V
2	19	V	V	A	V	V	V	V	A	V	V	A	V
3	8	V	V	V	V	V	V	V	V	V	V	A	V
4	6	A	V	V	V	V	V	A	V	V	A	V	V
5	5	A	V	V	V	V	V	V	V	V	V	V	V
6	4	A	V	A	V	V	V	V	V	V	V	V	V
7	3	A	V	V	V	V	V	V	V	V	A	V	V
8	3	V	V	A	V	V	V	V	V	V	A	V	V
9	3	V	V	V	V	V	A	V	V	V	V	V	V
10	2	V	V	A	V	V	V	V	V	V	V	V	V
11	2	V	A	V	V	A	A	V	V	V	V	V	V
12	1	V	V	V	V	V	V	V	A	V	V	V	V
13	2	A	V	A	V	V	V	V	A	V	A	V	V
14	2	V	V	V	V	V	V	V	A	V	V	A	V
15	1	V	V	A	A	V	V	A	V	V	V	V	V
16	1	V	A	A	V	A	V	V	V	V	V	V	V
17	1	V	V	A	V	A	V	V	V	V	V	V	V
18	1	A	V	A	V	A	V	V	V	V	V	A	V
19	1	V	V	V	A	A	V	V	V	V	V	V	V
20	1	V	A	V	V	A	A	A	V	V	V	V	V
21	1	A	A	A	V	V	A	V	V	V	V	V	V
22	1	V	A	V	A	A	V	V	A	V	V	V	V
23	1	A	A	V	V	V	V	V	V	V	A	V	V
24	1	A	V	A	V	V	V	A	A	V	A	V	V
25	1	V	V	A	V	A	A	A	V	V	V	A	V
26	1	A	V	A	V	V	A	V	V	V	A	A	A
27	1	V	V	V	A	V	V	V	V	V	V	V	V
28	1	V	V	V	V	V	V	V	V	V	A	A	V
29	1	A	V	A	V	V	V	A	V	V	A	V	V
30	1	A	V	V	V	V	A	V	V	A	A	V	A
31	1	A	V	A	V	V	V	V	V	A	V	V	V
32	1	V	A	V	V	V	V	V	V	A	A	V	V
33	1	A	A	V	V	V	V	V	V	A	A	V	V
34	1	V	V	V	V	A	V	V	A	V	V	A	V
35	1	V	V	A	V	V	V	V	V	A	V	V	A
36	1	A	A	A	A	V	A	V	V	A	V	V	A
37	7	V	V	A	V	V	V	V	V	V	V	V	A

^a VP-P = virulence phenotype of the parental isolate.

Table 2. Infection types (ITs) of the parental isolate (EK3-12) of *Puccinia striiformis* f. sp. *tritici* produced on 12 wheat *Yr* single-gene lines and segregations of avirulence (A) and virulence (V) phenotypes determined on 117 self-crossed progeny isolates

<i>Yr</i> gene line	IT of EK3-12	No. of progeny isolates		Expected ratio (A:V)	χ^2 ^a	<i>P</i> ^b	Virulence genes
		Avirulent	Virulent				
<i>Yr6</i>	9	30	87	1:3	0.90	0.01	<i>VYr6</i>
<i>Yr28</i>	3	28	89	1:3	0.76	0.09	<i>VYr28</i>
<i>Yr43</i>	9	30	87	1:3	0.90	0.01	<i>VYr43</i>
<i>Yr44</i>	4	27	90	1:3	0.60	0.27	<i>VYr44</i>
<i>Yr8</i>	4	55	62	9:7	0.04	4.06	<i>VYr8-1</i> , <i>VYr8-2</i>
<i>Yr7</i>	9	9	108	1:15	0.52	0.42	<i>VYr7-1</i> , <i>VYr7-2</i>
<i>Yr9</i>	9	5	112	1:15	0.38	0.78	<i>VYr9-1</i> , <i>VYr9-2</i>
<i>Yr17</i>	9	10	107	1:15	0.17	1.89	<i>VYr17-1</i> , <i>VYr17-2</i>
<i>Yr25</i>	9	10	107	1:15	0.30	1.06	<i>VYr25-1</i> , <i>VYr25-2</i>
<i>Yr27</i>	9	5	112	1:15	0.38	0.78	<i>VYr27-1</i> , <i>VYr27-2</i>
<i>Yr31</i>	9	5	112	1:15	0.38	0.78	<i>VYr31-1</i> , <i>VYr31-2</i>
<i>YrExp2</i>	9	11	106	1:15	0.16	1.99	<i>VYrExp2</i> , <i>VYrExp2-2</i>

^a χ^2 test for good fit of the observed numbers of progeny isolates in the avirulent and virulent groups to the theoretical ratios.

^b *P* value greater due to chance alone.

amplicons in the parental isolate and were codominant in the progeny isolates. Using the 10 markers, 75 MLGs were identified from the 117 progeny isolates (Supplementary Table S4). The heterozygosity of the 10 SSR loci varied from 52 to 58%, with a mean of 56%. All of the tested segregating SSR markers (RJ8 N, WSR23, WSR38, RJ24, RJ3, R10 N, RJ20, WSR54, WSR91, and PstP001) best fit the expected 1:2:1 homozygous (a1a1)/heterozygous (a1a2)/

homozygous (a2a2) ratio, with *P* values ranging from 0.18 to 0.66. Thus, these markers were suitable for molecular mapping. As an example, Figure 1 indicates that two alleles of the SSR marker WSR54 locus are in the parental isolate and are segregated among the progeny isolates.

A linkage map consisting of seven SSR markers spanning 245.6 cM was constructed (Fig. 2). The genetic distances ranged from

Fig. 1. Heterozygous and homozygous genotypes of simple sequence repeated marker WSR54 in the parental isolate and progeny isolates of *Puccinia striiformis* f. sp. *tritici*. **A**, Parental isolate EK3-12 and **B**, 1, **C**, 25, and **D**, 45 of progeny isolates.

19.7 cM between markers *WSR38* and *RJ10N* to 57.5 cM between *RJ8N* and *RJ24*. Sixteen QTLs for VPs corresponding to 11 *Yr* genes were mapped to the linkage map (Fig. 2; Table 3). The genetic positions of these QTLs started from 5 cM for *VYr25-1* to 245 cM for *VYr31-2* along the genetic map. Linkage relationships were determined for some of the virulence loci. *VYr25-1*, *VYr28-1*, and

VYr31-1 were mapped to the interval between SSR markers *WSR23* and *RJ8N*; *VYr27-1*, *VYr17-1*, and *VYr9-1* between *RJ24* and *PstP001*; *VYr28-2*, *VYr43*, *VYr44*, and *VYrExp2-1* between *PstP001* and *WSR38*; and *VYr7-1*, *VYr25-2*, *VYr17-2*, and *VYr31-2* between *RJ10N* and *WSR54*. The LOD values of the virulence loci ranged from 17.6 for *VYr8-1* to 89.1 for *VYr25-1*, and all were significant. The percentages of phenotypic variation explained by these QTLs ranged from 69.4% for *VYr27-1* to 97.5% for *VYr25-1* (Table 3). The allelic interactions were mostly dominant for virulence, consistent with the qualitative genetic analyses (Table 2).

Discussion

The discovery of alternate hosts of *P. striiformis* f. sp. *tritici* has made it possible to study inheritance of virulence (Rodríguez-Algaba et al. 2014; Tian et al. 2016; Wang et al. 2012, 2018; Yuan et al. 2018). In the present study, we produced 117 progeny isolates through self-fertilization of a single Ethiopian *P. striiformis* f. sp. *tritici* isolate on *B. holstii* under controlled greenhouse conditions, and we characterized them into 37 VPs and 75 MLGs. Through characterization of the parental isolate and progeny isolates on 22 *Yr* single-gene lines, we identified homozygous and heterozygous virulence/avirulence loci related to these *Yr* genes. Because the parental isolate represents the predominant races in Ethiopia, the identified homozygous avirulence loci corresponding to eight *Yr* genes (*Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *YrTr1*, *YrSP*, and *Yr76*) indicate the effectiveness of these resistance genes in Ethiopia. The segregations of avirulence phenotypes to three resistance genes (*Yr8*, *Yr28*, and *Yr44*) and of VPs to nine resistance genes (*Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, *Yr31*, *Yr43*, and *YrExp2*) led us to determine their inheritance and map the avirulence/virulence loci.

Sexual recombination could generate a large number of VPs and genotypes from a single highly dikaryotic (heterozygous) isolate or population. In the present study, we found that 53.9% of the progeny isolates had wider virulence spectra than the parental isolates. McDonald and Linde (2002) stated that populations of sexually produced pathogens usually exhibit a higher degree of genetic diversity than asexual reproductions. Other recent studies with *P. striiformis* f. sp. *tritici* also found a high number of VPs and genotypes through self-fertilization of single isolates on barberry plants (Tian et al. 2016; Wang et al. 2018; Yuan et al. 2018). These studies suggest that if sexual reproductions occur under natural conditions, *P. striiformis* f. sp. *tritici* is capable of producing a large number of races. Thus far, natural sexual reproduction of *P. striiformis* f. sp. *tritici* has been reported only in China but in very low frequencies (Wang et al. 2016; Zhao et al. 2013). More studies are needed to survey barberry plants

Fig. 2. A linkage map constructed with seven simple sequence repeated markers of 117 progeny isolates of *Puccinia striiformis* f. sp. *tritici* developed through sexual reproduction of Ethiopian isolate EK3-12 on *Berberis holstii* and quantitative trait loci mapping of virulence loci. LOD = logarithm of odds.

Table 3. Virulence loci and their genetic positions in the linkage map, simple sequence repeated (SSR) markers, logarithm of odds (LOD) values, percentages of phenotypic variation explained (PVE), and additive and dominance interactions

Virulence locus	Position	SSR marker		LOD	PVE (%)	Interaction	
		Left	Right			Additive	Dominance
<i>VYr25-1</i>	5	<i>WSR23</i>	<i>RJ8N</i>	89.1	97.5	0.0	-6.5
<i>VYr28-1</i>	30	<i>WSR23</i>	<i>RJ8N</i>	61.1	96.4	0.0	-5.7
<i>VYr31-1</i>	47	<i>WSR23</i>	<i>RJ8N</i>	45.5	86.3	0.0	-6.3
<i>VYr8-1</i>	60	<i>RJ8N</i>	<i>RJ24</i>	17.6	88.0	-0.3	-6.7
<i>VYr27-1</i>	108	<i>RJ24</i>	<i>PstP001</i>	21.5	69.4	0.2	-4.3
<i>VYr17-1</i>	151	<i>RJ24</i>	<i>PstP001</i>	47.8	90.3	0.0	-5.7
<i>VYr9-1</i>	153	<i>RJ24</i>	<i>PstP001</i>	49.2	88.8	0.0	-5.0
<i>VYr28-2</i>	171	<i>PstP001</i>	<i>WSR38</i>	57.2	96.4	0.0	-5.7
<i>VYr43</i>	171	<i>PstP001</i>	<i>WSR38</i>	32.0	91.3	-0.1	-6.8
<i>VYr44</i>	171	<i>PstP001</i>	<i>WSR38</i>	36.7	93.2	0.1	-5.2
<i>VYrExp2-1</i>	181	<i>PstP001</i>	<i>WSR38</i>	40.1	88.6	0.0	-4.1
<i>VYr9-2</i>	189	<i>WSR38</i>	<i>RJ10N</i>	46.5	88.8	0.0	-5.0
<i>VYr7-1</i>	210	<i>RJ10N</i>	<i>WSR54</i>	78.0	96.2	0.0	-8.6
<i>VYr25-2</i>	211	<i>RJ10N</i>	<i>WSR54</i>	88.7	97.5	0.0	-6.5
<i>VYr17-2</i>	241	<i>RJ10N</i>	<i>WSR54</i>	47.1	90.3	0.0	-5.7
<i>VYr31-2</i>	245	<i>RJ10N</i>	<i>WSR54</i>	45.7	86.2	0.0	-6.3

in Ethiopia and other countries to determine whether sexual production occurs in *P. striiformis* f. sp. *tritici* populations.

In the present study, the parental and progeny isolates were all homozygous avirulent to *Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *YrTr1*, *YrSP*, and *Yr76*. Similarly, homozygous avirulence loci to these genes, except *Yr76*, were also reported in previous studies with Chinese and U.S. isolates (Wang et al. 2018; Yuan et al. 2018). Homozygous avirulence loci to *Yr5* and *Yr15* were also observed in the study with another Chinese *P. striiformis* f. sp. *tritici* isolate (Tian et al. 2016). Thus far, there are no studies reporting heterozygous avirulence to *Yr5* and *Yr15*, which have been effective against almost all *P. striiformis* f. sp. *tritici* races in the world (Wan et al. 2017). From the mutation point of view, resistance genes, like *Yr5* and *Yr15*, should be relatively durable because their corresponding avirulence loci are homozygous compared with resistance genes with corresponding avirulence loci in the heterozygous state. Virulence change would happen more rapidly in a heterozygous population than a homozygous population because only one step of mutation in a heterozygous population is required for producing a VP (Yuan et al. 2018). Because we found that the avirulence locus to *YrSP* is homozygous in the isolate representing the predominant race and previous studies showed that *YrSP* was effective against all tested *P. striiformis* f. sp. *tritici* isolates from Ethiopia and Kenya (Sharma-Poudyal et al. 2013; Wan et al. 2017), *YrSP* should be useful for wheat breeding programs in these countries.

In the present study, we identified segregations of avirulence loci corresponding to three resistance genes (*Yr8*, *Yr28*, and *Yr44*) and virulence loci to nine resistance genes (*Yr6*, *Yr7*, *Yr9*, *Yr17*, *Yr27*, *Yr25*, *Yr31*, *Yr43*, and *YrExp2*). VPs for four resistance genes (*Yr6*, *Yr28*, *Yr43*, and *Yr44*) each were controlled by a single locus, whereas VPs for eight other resistance genes (*Yr7*, *Yr8*, *Yr9*, *Yr17*, *Yr25*, *Yr27*, *Yr31*, and *YrExp2*) were controlled by two loci. Thus, a total of 20 loci were identified through analyzing the avirulence/virulence data. Similar to our study, both a single locus and two loci for virulence were reported in previous studies (Tian et al. 2016; Wang et al. 2018; Yuan et al. 2018). Multiple avirulence genes corresponding to a single resistance gene have been reported in other plant pathogen systems. For example, Bourras et al. (2016) reported several avirulence genes corresponding to a single resistance gene in *Blumeria graminis* f. sp. *tritici*, the wheat powdery mildew pathogen.

Interestingly, the majority of avirulence phenotypes were recessive and VPs dominant in the present study. Although the segregation of avirulence to *Yr28* or *Yr44* statistically best fit a 1:3 ratio (Table 2), the best genetic model was not determined because the parental isolate had partially avirulent phenotypes. The inheritance of virulence to these two resistance genes could be more complex and requires further studies. In a study with a U.S. isolate, virulence was also dominant in all segregated loci (Yuan et al. 2018). In studies with Chinese isolates, virulence was dominant in the majority of segregated loci (Tian et al. 2016; Wang et al. 2018). Thus, dominant virulence is a common feature of inheritance for *P. striiformis* f. sp. *tritici*. The phenomenon of dominant virulence may be related to the nature of *P. striiformis* f. sp. *tritici* as an obligate biotrophic parasite, since the fungus completely relies on its host for growth, reproduction, and survival. In the absence of active gene products for virulence and the ability to overcome race-specific resistance genes, *P. striiformis* f. sp. *tritici* cannot infect and grow on the host plants (Wang et al. 2018). In contrast with this study, Tian et al. (2016) reported dominant avirulence to *Yr6*, *Yr43*, and *YrSP* in a sexual population produced from Chinese *P. striiformis* f. sp. *tritici* isolate Pinglan17-7. The differences could be attributable to the different isolates. These results indicate that avirulence can be either dominant or recessive depending on the isolates. Such a switch between dominance and recessiveness was reported in *P. triticina*, the wheat leaf rust pathogen. Different numbers and different inheritance modes were reported for avirulence to leaf rust resistance genes *Lr3*, *Lr10*, and *Lr30* using different progeny populations (Statler 2000; Statler and Jin 1991). A single dominant avirulence gene to *Lr3* and duplicate recessive genes for avirulence to *Lr30* were reported, but two independent dominant genes for avirulence to *Lr3* and

Lr30 genes were identified using different sexual populations. Statler (1982) identified a single dominant gene conditioning virulence to *Lr10*, but he later observed two dominant genes conditioning avirulence to the same resistance gene using a sexual population developed from a different isolate (Statler 2000). The different inheritance modes observed on sexually produced progeny isolates are probably attributable to different isolates that either have different genes conditioning avirulence/virulence, or some isolates have additional epistatic genes or entirely different genes conferring avirulence/virulence (Yuan et al. 2018). More studies are needed to understand complex interactions between different avirulence/virulence alleles in different isolates interacting with host resistance genes.

The interactions between *Puccinia* fungi and their hosts frequently fit the widely accepted gene-for-gene relationship, where the resistance phenotype of a host can be determined through the interaction between an avirulence (*Avr*) gene in the pathogen and its matching resistance gene in the host (Flor 1971). Resistance genes in the host plant probably encode putative receptors that respond to the product of an *Avr* gene expressed in the pathogen during the infection. Our study and other previous studies as discussed above confirmed that the relationship between a pathogen and its host is more complex than the early speculation that the two organisms provide products encoded by dominant genes for the interactions. Hence, further studies are needed to understand the mechanisms of complex interactions between *P. striiformis* f. sp. *tritici* and wheat.

In the present study, we developed a linkage map consisting of seven SSR markers. Although the number of markers was small, we were able to map 16 loci corresponding to 11 *Yr* genes using the QTL mapping approach. At least one locus for virulence was mapped for each of all 12 resistance genes to which the virulence/avirulence phenotypes segregated, except for *Yr6*. The genetic analysis of the phenotypic virulence data in the avirulence and virulence classes identified a total of 20 loci for virulence to 12 *Yr* genes, and the QTL analysis mapped 16 loci. Missing virulence loci was expected because of the small number of markers. The number of loci identified for virulence corresponding to each of the 12 *Yr* genes using both analyses was generally consistent. However, a discrepancy was found for *Yr28*. The VP to this resistance gene was controlled by a single locus, as the segregation of avirulence to virulence best fit a 1:3 ratio (Table 2). In contrast, the QTL mapping identified two loci, *VYr28-1* and *VYr28-2*, in the intervals between *WSR23* and *RJ8N* and between *PstP001* and *WSR38*, respectively, and more than 100 cM away (Table 3). The difference was likely attributable to the treatment of the phenotypic data. Because the IT data were lumped together into two classes (avirulent and virulent) in the segregation analysis, phenotype differences among the progeny isolates were reduced. On the other hand, QTL mapping utilized all different IT data, which detected the maximum phenotypic differences among the progeny isolates and therefore should produce more reliable results.

We identified four groups of virulence loci linked to each other. Linkages of virulence loci were found for *P. striiformis* f. sp. *tritici* in different studies (Tian et al. 2016; Wang et al. 2018; Yuan et al. 2018). Yuan et al. (2018) found that virulence genes to *Yr7* and *YrExp2* were linked to each other, similar to our study. Wang et al. (2018) also reported the linkage of virulence loci to *Yr17* and *Yr27*. Linkage of the virulence loci to *Yr27* and *Yr44* was reported by Tian et al. (2016). Thus, virulence genes tend to concentrate in some regions of the *P. striiformis* f. sp. *tritici* genome. Yuan et al. (2018) mapped 17 virulence loci on two of six linkage groups constructed with 805 GBS-SNP markers. In the present study, each group of the linked virulence loci was in a large genome region (>30 cM), owing to the small number of markers. Further studies are needed to verify the linkage, fill the genome regions with more markers, and clone the virulence genes. Nevertheless, the present study, together with previous studies, indicates that avirulence/virulence loci tend to be clustered in the *P. striiformis* f. sp. *tritici* genome.

In this study, we demonstrated that a single *P. striiformis* f. sp. *tritici* isolate was able to produce a large number of races through

sexual reproduction. Well-characterized virulence/avirulence genes can enhance understanding of wheat-*P. striiformis* f. sp. *tritici* interactions and can be used to guide breeding programs. The information obtained on the homozygosity of avirulence genes in the parental isolate representing the predominant race in Ethiopia is useful for breeding wheat cultivars for resistance to stripe rust. Resistance genes with corresponding homozygous avirulence loci should provide longer protection than those with corresponding heterozygous avirulence loci, as it will take longer for the pathogen to produce virulent races through mutation to overcome the former group resistance genes. In the present study, we found that the avirulence loci to *Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *YrTr1*, *YrSP*, and *Yr76* were homozygous in the parental isolate. Thus, these genes are more useful than the other resistance genes. Combining two or more genes, each of them is effective against all races, is better than combining genes, of which some have virulent races. Therefore, the results of this study, together with previous studies of virulence in the Ethiopian *P. striiformis* f. sp. *tritici* population (Wan et al. 2017), are useful for selecting effective resistance genes to be used in developing wheat cultivars with improved and durable resistance for sustainable control of stripe rust.

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