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RESEARCH ARTICLE

Determination of heterozygosity for avirulence/virulence loci through sexual hybridization of *Puccinia striiformis* f. sp. *tritici*

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Abstract Wheat stripe rust caused by *Puccinia striiformis* f. sp. *tritici* is one of the most devastating diseases of wheat worldwide and resistant cultivars are vital for its management. Therefore, investigating the heterozygosity of the pathogen is important because of rapid virulence changes in isolates heterozygous for avirulence/virulence. An isolate of *P. striiformis* f. sp. *tritici* was selfed on *Berberis shensiana* to determine the heterozygosity for avirulence/virulence loci. One hundred and twenty progeny isolates obtained from this selfing were phenotyped using 25 lines of wheat containing *Yr* genes and genotyped with 96 simple sequencing repeat markers, with 51 pathotypes and 55 multi-locus genotypes being identified. All of these were avirulent on lines with *Yr5*, *Yr10*, *Yr15*, *Yr24* and *Yr26* and virulent on lines with *Yr17*, *Yr25* and *YrA*, indicating that the parental isolate was homozygously avirulent or homozygously virulent for these loci. Segregation was found for wheat lines with *Yr1*, *Yr2*, *Yr4*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr27*, *Yr28*, *Yr32*, *Yr43*, *Yr44*, *YrExp2*, *YrSp*, *YrTr1*, *YrTye* and *YrV23*. The 17 cultivars to which the *Pst* was identified as heterozygous with respect to virulence/avirulence should not be given priority in breeding programs to obtain new resistant cultivars.

Keywords *Puccinia striiformis* f. sp. *tritici*, selfing, heterozygosity, virulence inheritance

1 Introduction

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*) causes significant yield losses during epidemics that have resulted in famines throughout human history[1]. The application of resistant cultivars is an effective way to control *Pst*. However, resistance in cultivars is usually not durable, and the appearance of *Pst* races that overcome widely deployed resistance (R) genes has led to destructive pandemics in the past. New races continuously appear due to mutation, introduction, genetic drift, asexual and/or sexual recombination, and host selection[2–7]. Most changes in virulence of *Melampsora lini* isolates, for example, were found to be due to mutations followed by sexual recombination[8]. Studying the level of heterozygosity in *Pst* seems appropriate because virulence changes take place more rapidly in isolates heterozygous for avirulence than in isolates homozygous for avirulence[9]. High genome heterozygosity has been shown for *Pst* based on molecular markers, and allelic and genomic sequences[1,10,11]. Direct evidence for virulence/avirulence heterozygosity can be acquired from a selfing or crossing experiment on an alternate host[12].

Pst is a heteroecious pathogen, and five spore stages represented by urediospores, teliospores, basidiospores, pycniospores and aeciospores are included in its life cycle, with the first two stages present on wheat and the rest on an alternate host[13]. Crossing experiments in the greenhouse indicated that new races of *Pst* could be produced on barberry (*Berberis* spp.)[14,15]. However, barberry and Oregon grape (*Mahonia* spp.) were only recently confirmed as alternate hosts for *Pst*[16,17]. Heterozygosity, inheritance of avirulence/virulence, and linkage relationships of genes for pathogenicity may be acquired using long-established genetic approaches including selfing and

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hybridization of isolates[9,18–20]. The confirmation of *Berberis* spp. as alternate hosts for *Pst* made it possible to study the genetics of virulence of *Pst* by selfing and crosses. The inheritance of virulence/avirulence of *Pst* has been studied by Wang et al.[21] in the USA, Rodriguez-Algaba et al.[22] in Europe and Tian et al.[12] in China.

The gene-for-gene relationship implies that the pathogenicity genotype of a pathogen isolate can be determined through studying the infection patterns of its progeny isolates on host lines harboring different resistance genes[23]. A set of near-isogenic lines of wheat genotypes with an "Avocet Susceptible" background was found to be useful in distinguishing races of *Pst* except for ones which cannot infect Avocet S[24]. Resistance genes encode putative receptors that respond to the products of '*Avr* genes' (*Avr*, avirulence) which are a subset of pathogen-secreted virulence proteins produced by the pathogen during infection[25,26].

In this study, a sexual population comprising 120 isolates produced by selfing of an isolate collected from Gansu Province, China with a relatively wide virulence spectrum was built. Virulence tests and SSR genotypes were used to determine inheritance and heterozygosity of avirulence/virulence loci corresponding to various *Yr* genes in the population. The data obtained on the heterozygosity of *Pst* may help to predict virulence changes in rust populations, which could prove significant in the decision making process for the generation and distribution of new resistant wheat cultivars.

2 Materials and methods

2.1 Urediospore multiplication and teliospore production

The parental isolate was collected from Gansu Province, China in 2013. Urediospores were multiplied from a single urediospore on susceptible wheat cv. Mingxian 169 with no known resistance genes to *Pst*. Wheat seedlings were grown in 10 cm square pots. When the first few leaves were fully expanded, seedlings were inoculated with a spore suspension (0.1 mg·mL^{-1})[22], and incubated for 24 h at 10°C in the dark in a dew chamber. Afterwards, the plants were transferred to a growth chamber at 16°C with a 16L:8D h photoperiod. Teliospores were produced by inoculating urediospores onto vegetative plants at the 5 leaf stage. When heavy sporulation became visible, the temperature was raised to 25°C during the day and 16°C at night to induce telia formation. Teliospore-producing leaves were harvested 1 month after inoculation. Leaves were dried at room temperature for 2 days, put in paper bags, and kept in a desiccator at 4°C for about 1 week.

2.2 Inoculation of barberry

Seedlings of *Berberis shensiana* for inoculation were grown in pots in the greenhouse. Teliospores were germinated following the method described by Zhao et al.[27]. After abundant basidiospore production was observed, these were used for inoculation as detailed by Tian et al.[12]. All further steps were described by Tian et al.[12].

2.3 Selfing of the parental isolate

After appearance of nectar on the pycnia, nectar was transferred from one pycnium to another with a toothpick that had been soaked in sterile water for several hours. Aecia appeared at 5–7 days after crossing.

2.4 Development of uredial progeny

Aeciospores were collected from barberry leaves carrying mature aecia by gentle tapping above a microscope slide. A single aeciospore was picked with a fine glass fiber under the stereomicroscope and used to inoculate a seedling of wheat cv. Mingxian 169 at the one-leaf stage. Prior to inoculation, the wax layer on the leaves was removed. Inoculated plants were kept at 10°C in a dew chamber for 24 h in the dark. Afterwards plants were transferred to a growth chamber at 16°C with a 16L:8D h photoperiod. Plants successfully inoculated in one pot were separated to new pots to avoid contamination. After 15 days of incubation, urediospores produced on a single leaf were collected with a glass fiber and stored in a desiccator at 4°C for later use.

2.5 Virulence assays

The parental isolate and progeny isolates were tested for pathogenicity on a set of 25 wheat genotypes, most of which were near-isogenic lines from an Avocet-susceptible background (Table S1). Cultivar Mingxian 169 was used as susceptible control. The procedures and conditions for inoculation and cultivation were as previously described[28]. Infection types (ITs) were scored 18–20 days after inoculation based on a 0–9 scale as described by Line and Qayoum[29]. ITs 0–5 were considered avirulent (A) and ITs 6–9 virulent (V). Virulence phenotyping was repeated twice for all isolates. For genetic analysis, the progeny isolates produced by selfing were considered as F_2 generation, and the heterokaryotic urediospores were considered as diploid. X^2 tests were used to determine the degree of fit of observed numbers to theoretical segregation ratios for both virulence and SSR markers. The number of virulence phenotypes (VPs), that is pathotypes, was determined using VAT 1.0 software[30].

2.6 DNA extraction

DNA was extracted from urediospores using the method described by Aljanabi and Martinez[31] with modifications.

Urediospores were transferred to a 1.5 mL centrifuge tube, sterilized silica sand equivalent to the spore volume and 200 μL extraction buffer (0.4 mol·L^{-1} NaCl; 10 mmol·L^{-1} Tris-HCl, pH 8; 2 mmol·L^{-1} EDTA pH 8) were added, and the mixture was ground with a plastic pestle for 10–15 min. After adding another 300 μL of extraction buffer and 5 μL of 10 μg·μL^{-1} proteinase K and gentle mixing, the mixture was incubated at 65°C for 2 h. A standard phenol-chloroform-isoamyl alcohol extraction followed and the dried DNA pellet was finally dissolved in 50 μL TE buffer. 1.0 mL of 20 μg·mL^{-1} RNase A was added and the mixture incubated at 37°C for 30 min. RNase was removed by phenol-chloroform-isoamyl alcohol extraction and purified DNA was dissolved in TE buffer. DNA concentration was determined with an ND-1000 spectrophotometer (Bio-Rad, Martinez, CA, USA), and the concentration was adjusted to 50 ng·mL^{-1}.

2.7 SSR primers, PCR amplification, electrophoresis and data analyses

Ninety six pairs of *Pst* SSR primers[32–38] (Table S2) were screened for useful markers. Forward primers were fluorescently 5′-labeled with either FAM, HEX, TAM or ROX. Primers were synthesized by Shanghai Sangon Company (Shanghai, China). PCR was performed in a S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a 25 μL volume comprised of 1.0 μL of DNA, 2.5 μL of 10 × reaction buffer (Mg^{2+}-free), 2.0 μL of Mg^{2+} (25 mmol·L^{-1}), 0.5 μL of dNTPs (10 mmol·L^{-1}), 0.5 μL of each primer (10 mmol·L^{-1}), 0.2 μL Taq DNA polymerase (5 U·μL^{-1}, Takara Bio Inc., Kusatsu, Japan) and 17.8 μL of ddH_2O. PCR was carried out under the following conditions: initial denaturation at 95°C for 3 min; 10 cycles at 95°C for 30 s, 63°C for 30 s with a 1°C decrease per cycle, and 72°C for 30 s; 20 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 6 min. PCR products were analyzed on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Pittsburgh, PA, USA). The lengths of the SSR amplicons were scored using GeneMarker[39]. The correlation coefficient of virulence phenotypes and SSR genotypes was calculated using NTSYS-pc (V2.10) MXCOMP[40]. Linkage maps were constructed using QTL IciMapping software (V4.1)[41].

3 Results

3.1 Segregation of virulence

Fifty one VPs were found based on virulence tests of 120 isolates used in this study (Table 1; Table S3). Segregation of virulence was found for 17 of the 25 wheat lines (Table 1). For correspondence with the host genes, the codes *CAvr1*, *CAvr2*, *CAvr4*, *CAvr5*, *CAvr6*, *CAvr7*, *CAvr8*, *CAvr9*, *CAvr10*, *CAvr15*, *CAvr17*, *CAvr24*, *CAvr25*, *CAvr26*, *CAvr27*, *CAvr28*, *CAvr32*, *CAvr43*, *CAvr44*, *CAvrA*, *CAvrExp2*, *CAvrSp*, *CAvrTr1*, *CAvrTye* and *CAvrV23* are assigned to the genes for avirulence; *Cavr1*, *Cavr2*, *Cavr4*, *Cavr5*, *Cavr6*, *Cavr7*, *Cavr8*, *Cavr9*, *Cavr10*, *Cavr15*, *Cavr17*, *Cavr24*, *Cavr25*, *Cavr26*, *Cavr27*, *Cavr28*, *Cavr32*, *Cavr43*, *Cavr44*, *CavrA*, *CavrExp2*, *CavrSp*, *CavrTr1*, *CavrTye* and *CavrV23* are assigned to the genes for virulence. VP 1 comprised 26 isolates having the same virulence/avirulence pattern as the parental isolate (Table 1). All other VPs contained one to eight isolates. Fifteen VPs containing 42 isolates showed a higher degree of virulence compared to the parental isolate. Seven of these 15 VPs comprising 15 isolates had a wider spectrum of virulence compared to the parental isolate. These isolates had additional virulence on one or several loci of *Yr4*, *Yr7*, *Yr8*, *Yr32*, *YrExp2* and *YrSp*. VP 10 consisting of only isolate 111 infected 17 of the 25 wheat lines with *Yr* genes. Twenty six VPs comprising 59 isolates showed a decreased frequency of virulence. The remaining 10 VPs showed the same frequency of virulence as the parental isolate. Taken together, these results indicate that a large number of races with a wide range of virulence can be generated through sexual reproduction.

The parental isolate and all S1 progeny isolates were avirulent on lines with *Yr5*, *Yr10*, *Yr15*, *Yr24* and *Yr26*. This indicates that the parental isolate was homozygously avirulent. On lines with *Yr17*, *Yr25* and *YrA*, the parental isolate and all S1 progeny isolates were virulent. This in turn shows that the parental isolate was homozygous with respect to this group of resistance genes.

Segregation for pathogenicity among S1 progeny isolates occurred on 17 lines with *Yr* resistance genes (*Yr1*, *Yr2*, *Yr4*, *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr27*, *Yr28*, *Yr32*, *Yr43*, *Yr44*, *YrExp2*, *YrSp*, *YrTr1*, *YrTye* and *YrV23*). The parental isolate was avirulent on lines with *Yr4*, *Yr7*, *Yr8*, *Yr32*, *Yr43*, *YrExp2*, *YrSp* or *YrTr1*, but virulent on lines with *Yr1*, *Yr2*, *Yr6*, *Yr9*, *Yr27*, *Yr28*, *Yr44*, *YrTye* or *YrV23*. The best fit for the segregation of S1 progeny isolates was a 3:1 avirulence to virulence ratio for the reaction on *Yr7*, *Yr8*, *YrExp2* and *YrSp*, and 15:1 for the reaction on lines with *Yr4*, *Yr32* and *YrTr1* (Table 2). Two examples of different reactions produced by the parental and some progeny isolates are illustrated in Fig. 1. The best fit for the virulence on lines with *Yr1* or *Yr27* was a 1:3 avirulence to virulence ratio and 1:15 for the reaction on the line with *Yr6*. Since the *P* values were all > 0.05, these data suggest single recessive *Pst* genes for virulence (or avirulence) in relation to *Yr7*, *Yr8*, *YrExp2*, *YrSp*, *Yr1* and *Yr27*. By contrast, two complementary recessive genes for virulence (or two independent dominant genes for avirulence), or avirulence (or two independent dominant genes for virulence) are indicated for the reaction on lines with *Yr4*, *Yr32*, *YrTr1* and *Yr6*. The best fit for the segregation of S1 progeny isolates was a 13:3 avirulence to virulence

Table 1 Virulence patterns of the parental and progeny isolates of *Pst* on specific wheat *Yr* lines

VP	Number of isolate	Avirulence (A) and virulence (V) of parental and progeny isolates of *Pst* on wheat lines with *Yr* genes																
		Yr4	*Yr7*	*Yr8*	*Yr32*	*Yr43*	*YrExp2*	*YrSp*	*YrTr1*	*Yr1*	*Yr2*	*Yr6*	*Yr9*	*Yr27*	*Yr28*	*Yr44*	*YrTye*	*YrV23*
Parent	1	A	A	A	A	A	A	A	A	V	V	V	V	V	V	V	V	V
Progeny	120																	
1	26	A	A	A	A	A	A	A	A	V	V	V	V	V	V	V	V	V
2	6	A	A	A	A	A	V	A	A	V	V	V	V	V	V	V	V	A
3	1	A	A	A	A	A	V	A	A	V	V	V	V	V	V	V	V	V
4	1	A	A	A	V	A	A	A	A	V	V	V	V	V	V	V	V	V
5	1	A	A	A	A	A	A	A	A	V	V	V	V	V	V	V	A	V
6	1	A	A	A	A	A	A	A	A	V	V	V	A	V	V	V	V	V
7	1	A	A	V	A	A	A	A	A	V	A	V	V	V	V	V	V	V
8	6	A	A	A	A	A	A	A	A	V	A	V	V	V	V	V	V	V
9	8	A	A	A	A	A	A	V	A	V	V	V	V	V	V	V	V	V
10	1	V	V	A	V	A	A	V	A	V	V	V	V	V	V	V	V	V
11	3	A	A	A	A	A	A	V	A	V	A	V	V	V	V	V	V	V
12	2	V	A	A	A	A	A	V	A	V	V	V	V	V	V	V	V	V
13	1	V	A	A	A	A	A	A	A	V	V	V	V	A	V	V	V	V
14	1	A	A	A	A	A	A	A	A	A	V	V	V	A	V	V	V	V
15	1	A	V	A	A	A	A	A	A	V	V	V	V	V	V	A	V	V
16	1	A	V	A	A	A	A	A	A	V	V	V	V	V	V	V	V	V
17	3	A	A	A	A	A	A	A	A	V	A	V	V	V	V	A	V	V
18	2	A	A	A	A	A	A	A	A	V	V	V	V	V	V	A	V	V
19	1	A	A	A	A	A	A	A	A	V	A	V	V	A	V	A	V	V
20	4	A	A	A	A	A	A	V	A	V	A	V	V	V	V	V	V	A
21	2	A	A	V	A	A	A	V	A	V	A	V	V	V	V	V	V	A
22	1	A	A	A	A	A	A	A	A	V	A	V	V	V	V	A	V	A
23	7	A	A	A	A	A	A	A	A	V	A	V	V	V	V	V	V	A
24	1	A	A	A	A	A	A	A	A	V	A	V	V	A	V	V	V	A
25	1	A	A	A	A	A	A	A	A	V	A	V	V	V	V	V	A	A
26	1	A	A	A	A	A	A	V	A	V	A	V	V	V	V	A	V	A
27	1	A	A	A	A	A	A	V	A	V	A	A	V	V	V	A	V	A
28	1	A	A	A	A	A	A	A	A	A	A	V	V	A	V	A	V	A

(*Continued*)

VP	Number of isolate	Avirulence (A) and virulence (V) of parental and progeny isolates of *Pst* on wheat lines with *Yr* genes																
		Yr4	*Yr7*	*Yr8*	*Yr32*	*Yr43*	*YrExp2*	*YrSp*	*YrTr1*	*Yr1*	*Yr2*	*Yr6*	*Yr9*	*Yr27*	*Yr28*	*Yr44*	*YrTye*	*YrV23*
29	2	A	A	A	A	A	A	A	A	A	A	V	V	V	V	A	V	A
30	1	A	A	V	A	A	V	A	A	V	V	V	V	V	V	V	V	A
31	1	A	A	V	A	A	V	A	A	V	V	V	V	V	V	V	V	V
32	1	A	A	V	A	A	A	A	V	V	V	V	V	V	V	V	V	A
33	1	A	A	V	A	A	A	V	A	V	A	V	V	V	V	A	V	V
34	1	A	A	A	V	V	A	A	A	V	V	V	V	V	A	V	V	V
35	1	A	A	V	A	A	A	A	A	A	A	V	A	V	V	V	V	V
36	1	A	V	A	A	A	A	A	V	V	V	V	V	V	A	V	V	A
37	1	A	V	A	A	A	V	A	V	V	V	V	V	V	A	V	V	A
38	1	A	V	V	A	A	A	V	A	V	A	V	V	V	V	V	A	A
39	1	A	A	V	A	A	V	A	A	A	V	A	V	A	V	V	V	A
40	1	A	V	A	V	A	V	V	A	A	V	A	V	V	V	V	V	V
41	1	V	V	A	A	A	A	V	A	V	V	A	A	V	V	A	A	V
42	1	A	V	V	A	V	V	A	A	V	V	V	A	A	A	V	V	A
43	2	A	V	V	A	V	V	A	A	A	V	V	A	A	V	V	A	A
44	8	A	V	V	A	V	V	A	A	A	V	V	A	A	A	V	A	A
45	3	A	V	V	A	V	V	A	V	A	V	V	A	A	A	V	A	A
46	1	A	V	V	A	V	V	A	V	A	A	V	A	A	A	V	V	A
47	1	A	V	V	A	V	V	A	V	A	V	V	A	A	V	V	V	A
48	1	A	V	V	V	V	V	A	A	V	V	V	V	V	A	V	A	A
49	1	V	V	V	V	V	V	A	A	V	V	V	V	V	V	V	V	A
50	1	A	V	V	A	A	V	A	A	V	A	A	V	V	A	V	A	A
51	1	A	V	V	A	V	V	A	A	V	V	A	A	V	A	V	A	A

Note: VP, Virulence phenotype. Progeny isolates were separated on 17 of the 25 *Yr* lines. The parental isolate and progeny isolates were all homozygously avirulent on *Yr5*, *Yr10*, *Yr15*, *Yr24*, and *Yr26* and homozygously virulent on *Yr17*, *Yr25*, and *YrA*.

Table 2 Infection types, segregation of avirulence/virulence in the progeny isolates derived from selfing parental *Pst* isolate on wheat lines with the indicated *Yr* genes and probability (*P*) values of Chi-square tests

Wheat line with *Yr* gene	Infection type of parental isolate	Observed number of progeny isolates		Expected ratio	*P*
		Avirulent	Virulent		
Yr7	2	92	28	3:1	0.67
Yr8	0	90	30	3:1	1.00
Yr32	1	115	5	15:1	0.35
Yr43	2	100	20	13:3	0.56
YrSp	0	94	26	3:1	0.40
YrTr1	2	112	8	15:1	0.85
YrExp2	2	94	26	3:1	0.40
Yr4	2	115	5	15:1	0.35
Yr1	9	21	99	1:3	0.73
Yr2	9	40	80	7:9	0.03
Yr6	9	6	114	1:15	0.19
Yr9	9	20	100	3:13	0.56
Yr27	9	22	98	1:3	0.09
Yr44	9	15	105	3:13	0.08
YrTye	9	20	100	3:13	0.56
Yr28	9	19	101	3:13	0.41
YrV23	9	53	67	7:9	0.93

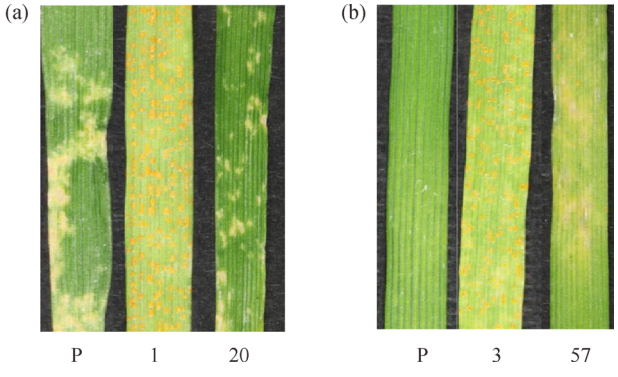

Fig. 1 Infection types (IT) of the parental isolate (P) and its selfed progeny isolates (1, 3, 20, and 57) on wheat near-isogenic lines of *Yr7* (a) and *Yr8* (b).

ratio for the line with *Yr43* and 3:13 for lines with *Yr9*, *Yr28*, *Yr44* and *YrTye*. These ratios indicate the presence of two recessive genes. This could be explained by epistatic interaction of one gene dominant for avirulence and one gene dominant for suppression of avirulence. The best fit for segregation on phenotypes *Yr2* and *YrV23* was a 7:9 avirulence to virulence ratio when two dominant gene pairs exist conditioning virulence. However, the *P* value of the ratio on *Yr2* was below 0.05.

Chi-square tests for independence indicated linkage between pathogen genes C*Avr7*, C*Avr8* and C*AvrExp2*, and between C*avr1* and C*avr27* (Table 3). The linkage relationship indicated that C*Avr7*, C*Avr8* and *CAvrExp2* may be clustered at a specific location.

Table 3 Probability (*P*) values of Chi-square tests for independence of genes for avirulence/virulence

CAvr or cavr gene	*P*			
	Avirulence (*CAvr*)			Virulence (*Cavr*)
	CAvr8	*CAvrExp2*	*CAvrSp*	*Cavr27*
CAvr7	0.00	0.00	0.61	
CAvr8		0.00	0.53	
CAvrExp2			0.10	
Cavr1				0.00

Note: $P \geqslant 0.05$ indicates that the two genes are independent and not linked, and $P < 0.05$ indicates that the two genes are linked.

3.2 Segregation of SSR markers and linkage map

Of the 96 SSR markers used in this study, 11 produced co-dominant amplicons in the parental isolate that segregated among the selfed progeny population. Fifty five multilocus genotypes were identified from the 120 progeny isolates (Table S4). A linkage map that contains four avirulence genes and 11 SSR markers was built using QTL Icimapping software (Fig. 2). The dominant avirulence genes, *CAvr7*, *CAvr8* and *CAvrExp2,* were more closely linked than *CAvrSp*. The correlation coefficient between virulence phenotype and SSR genotype was –0.09.

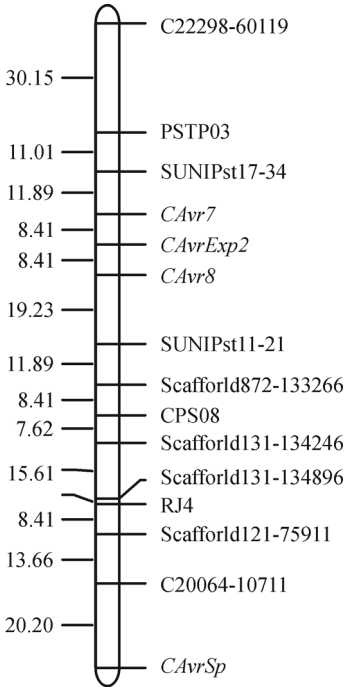

Fig. 2 Linkage maps constructed with SSR markers and avirulence data of 120 progeny isolates developed through selfing using QTL IciMapping software

4 Discussion

In this study, a *Pst* population containing 120 isolates acquired from selfing a single parental isolate on *B. shensiana* was built. Virulence tests and SSR molecular markers were used to analyze the population. Segregation of virulence was found for 17 of the 25 virulence/avirulence loci. A linkage map including four avirulence genes and 11 SSR molecular markers was built.

From the 120 progeny isolates, 51 VPs and 55 genotypes were identified. This indicates that sexual reproduction can produce isolates with a higher diversity than asexual reproduction. Among the 51 VPs, seven VPs containing 15 isolates had an even wider spectrum than the parental isolate for one or several loci of *Yr4*, *Yr7*, *Yr8*, *Yr32*, *YrExp2* and *YrSp*. These 15 isolates are likely to be the dominant races in the future. This work constitutes a novel approach that might be used in the future to predict the appearance of novel races.

Our data are largely compatible with previous studies of selfing a *Pst* isolate PST-127 from the USA, and isolate Pinglan 17-7 from China[12,21] (Table 4). However, in the present study, we found avirulence on the wheat line with *Yr1* is controlled by a single recessive gene, which was previously identified as homozygously virulent by Tian et al.[12] and Wang et al.[21]. However, the new data indicate that avirulence on lines with *Yr2* and *Yr9* seems to be controlled by two genes each, and therefore cannot be considered homozygous for virulence in PST-127 and Pinglan 17-7[12,21]. Avirulence on the line with *Yr4* seems to be controlled by two dominant genes instead of two recessive genes in Pinglan 17-7[12]. Avirulence on the line with *Yr6,* on the other hand, seems to be controlled by two recessive genes, not by a single dominant gene in PST-127 and Pinglan 17-7. Avirulence on lines with *Yr7* and *Yr8* is controlled by single dominant genes, which corresponds to the results by Wang et al.[21], but not the results by Tian et al.[12]. Avirulence on the line with *Yr27* in our case seems to be controlled by one recessive gene, as in the study of Tian et al.[12], however, Wang et al.[21] had identified a single dominant gene. Avirulence on the line with *Yr32* is controlled by two dominant genes, and not controlled by two recessive genes in isolate Pinglan 17-7[12] and homozygous avirulence in race PST-127[21]. Avirulence against *YrTr1* was controlled by two dominant gene, not homozygous avirulence to *YrTr1* in isolate Pinglan 17-7[12] and controlled by one recessive genes in race PST-127[21]. Avirulence against *YrSp* was controlled by a dominant gene in the present study and Pinglan 17-7 [12] but homozygous in race PST-127[21]. Avirulence against *YrExp2* was controlled by one dominant gene in the present study and PST-127[21] but homozygous in Pinglan 17-7[12]. Avirulence against *Yr28*, *Yr43*, *Yr44*, *YrTye* and *YrV23* was controlled by two genes in the present study but this was not the same for Pinglan 17-7 or PST-127[12,21]. These inconsistencies indicate that avirulence/virulence against a single *Yr* gene can be either dominant or recessive, and can be controlled by different genes in different segregating populations.

Segregation for virulence was found for 17 avirulence/virulence loci. Segregation on lines with *Yr2*, *Yr4*, *Yr32*, *YrTr1*, *Yr6, Yr43*, *Yr9*, *Yr28*, *Yr44*, *YrTye* and *YrV23* suggests that there are two genes controlling avirulence or virulence. However, this is inconsistent with the gene-for-gene concept. As genes for resistance in near-isogenic lines should correspond to genes for pathogenicity in the pathogen[23]. However, the reaction to some resistance genes might be controlled by multiple genes as indicated by work on wheat brown rust and barley powdery mildew[9,42,43]. There may be two reasons for this: (1) an additional gene for resistance in these cultivars, or two genes for virulence were acquired by *Pst* to overcome the

Table 4 Expected avirulence to virulence ratios of wheat lines with different *Yr* gene in different sexually reproducing populations

Yr gene in the tested wheat line	Avirulence to virulence ratios		
	Present study	PST-127	Pinglan 17-7
Yr1	1: 3	Homozygously virulent	Homozygously virulent
Yr2	7: 9	Homozygously virulent	Homozygously virulent
Yr4	15: 1	–	1: 15
Yr5	Homozygously avirulent	Homozygously avirulent	Homozygously avirulent
Yr6	1: 15	3: 1	3: 1
Yr7	3: 1	3: 1	Homozygously virulent
Yr8	3: 1	3: 1	Homozygously avirulent
Yr9	3: 13	Homozygously virulent	Homozygously virulent
Yr10	Homozygously avirulent	1: 3	Homozygously virulent
Yr15	Homozygously avirulent	Homozygously avirulent	Homozygously avirulent
Yr17	Homozygously virulent	1: 3	Homozygously virulent
Yr24	Homozygously avirulent	Homozygously avirulent	Homozygously virulent
Yr25	Homozygously virulent	–	Homozygously virulent
Yr26	Homozygously avirulent	–	Homozygously virulent
Yr27	1: 3	3: 1	1: 3
Yr28	3: 13	–	1: 3
Yr32	15: 1	Homozygously avirulent	1: 15
Yr43	13: 3	3: 1	3: 1
Yr44	3: 13	3: 1	1: 15
YrA	Homozygously virulent	–	Homozygously virulent
YrExp2	3: 1	3: 1	Homozygously virulent
YrSp	3: 1	Homozygously avirulent	3: 1
YrTr1	15: 1	1: 3	Homozygously avirulent
YrTye	3: 13	3: 1	–
YrV23	7: 9	–	Homozygously virulent

Note: PST-127 and Pinglan 17-7 were isolates used in previous study for inheritance of *Pst*[12,21].

single resistant gene; or (2) the presence of an additional modifier gene[9,43,44]. Avirulence against *Yr2* favored a 7:9 ratio over other ratios ($P < 0.05$), this may be due to the limited numbers of progeny.

Linkage between avirulence genes has been observed in *M. lini*, *P. recondita* and *P. graminis*, though not frequently[9,18,43,45]. In the present study, from the linkage map we found both *CAvr7* and *CAvr8* linked to *CAvrExp2* within 8.41 cM. *CAvrrSp* on the other hand seems independently inherited. Close genetic linkage was also found for *Cavr1* and *Cavr27*. Linkage between *Avr6* and *Avr43* was found within 7.37 cM in a previous study[12]. This is contrary to a study on *P. recondita*, where almost all avirulence/virulence loci segregated independently and did not cluster at specific locations[44].

It has been reported that there is a substantial amount of heterozygosity in *P. recondita*[9,18]. In this study, we found the same for *Pst*. Segregation was found on 17 of 25 marker loci, which indicates that the parental isolate was heterozygous at these 17 loci. Investigating the heterozygosity in rust fungi is useful to predict virulence changes in rust populations[13]. Heterozygosity probably leads to an increase in pathogenic variation in natural *Pst* populations, as virulence changes will occur in heterozygous populations more rapidly than in a homozygous ones[18]. The use of resistant cultivars is an effective way to control *Pst*. Knowledge about homozygosity or heterozygosity for avirulence/virulence genes is therefore a prerequisite for successful resistance breeding. Avirulence to *Yr5* and *Yr15* was homozygous in the present study and in a previous one[12]. This result is in accordance with the effectiveness of these resistance genes at present in China and the USA[46]. Avirulence to *Yr10*, *Yr24* and *Yr26* was homozygous in this study. However, virulence to *Yr10*, *Yr24* or *Yr26* has been identified in the *Pst* population in China[47,48]. This may be due to only one *Pst* isolate being

used in this study. More isolates representing diverse races should be studied to improve knowledge of homozygosity or heterozygosity for avirulence/virulence genes in natural *Pst* populations. Avirulence to *Yr24*, *Yr32* and *YrSp* was homozygous in PST-127 from America, and frequencies of virulence toward these resistance genes were low in USA[21,46]. The 17 cultivars identified as heterozygous with respect to virulence/avirulence should not be given priority in breeding programs to obtain new resistant cultivars. For breeding programs, it is better to combine resistance genes that are still effective against all known races, and to combine effective all-stage resistance with durable adult-plant resistance genes[49].

Segregation was only found for 11 of 96 markers selected for this study. Few SSR markers presenting diversity were also found in previous studies[12,22]. The correlation coefficient between virulence phenotype and SSR genotype was low. The low correlation indicated that SSR markers were not based on virulence genes. The limited SSR markers that present diversity in the population of *Pst* showed that SSR markers were not a good choice for mapping of avirulence genes in *Pst*. Other molecular markers presenting high diversity between progeny of sexual reproduction should be used in the study on mapping of avirulence genes. This will lay a foundation for avirulence/virulence genes cloning using a map-based cloning approach.

5 Conclusions

Virulence tests and SSR genotypes were used to analyze a population produced by selfing an isolate collected from Gansu Province, China to determine inheritance and heterozygosity of avirulence/virulence loci corresponding to various *Yr* genes. Seventeen of the 25 loci tested in this study were heterozygous. Avirulence/virulence on wheat cultivars with *Yr* genes was controlled by one or two genes. The linkage relationship indicated that C*Avr7*, C*Avr8* and *CAvrExp2* may be clustered at a specific location. These data on heterozygosity of avirulence/virulence loci will be useful in the development and distribution of new resistant wheat cultivars.

Supplementary materials The online version of this article at http://dx.doi.org/10.15302/J-FASE-2016114 contains supplementary materials (Tables S1–S4).

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