Virulence characteristics of *Blumeria graminis* f. sp. *tritici* and its genetic diversity by EST-SSR analyses

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Abstract: Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (an obligate biotrophic pathogen) is a worldwide threat to wheat production that occurs over a wide geographic area in China. Monitoring genetic variation and virulence structure of the pathogen are necessary for effective disease resistance breeding and significant implications for agronomic control of wheat powdery mildew. Results indicated that 90% of all isolates were virulent on *Pm3c*, *Pm3e*, *Pm3f*, *Pm4a*, *Pm5*, *Pm6* (Tingalen), *Pm7*, *Pm16*, *Pm19*, and *Pm1 + 2 + 9* and 62.6% to 89.9% of isolates were virulent on *Pm2*, *Pm3a*, *Pm3b*, *Pm3d*, *Pm4b*, *Pm6* (Coker747), *Pm8*, *Pm17*, *Pm20*, *Pm23*, *Pm24*, *Pm30*, *Pm4+8*, *Pm5+6*, *Pm4b + mli*, *Pm2 + mld*, *Pm4 + 2X*, *Pm2 + 6*. The *Pm13* and *PmXBD* genes were effective against most collected isolates from Liaoning and Heilongjiang Provinces. Only *Pm21* exhibited an immune infection response to all isolates. Furthermore, closely related isolates within each region were distinguished by cluster analyses using EST-SSR representing some gene

exchanges and genetic relationships between the flora in Northeast China (Liaoning, Heilongjiang) and Sichuan. Only 50% of the isolates tested show a clear correlation between EST-SSR genetic polymorphisms and the frequency of virulence gene data.

Keywords: *Blumeria graminis* f. sp. *tritici*; wheat powdery mildew; resistance; *Pm* genes

Introduction

Wheat powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is one of the major diseases affecting the production of wheat in China. *Bgt* is widely distributed and displays a complex population structure and rapid mutation rate, which make the disease extremely virulent and difficult to prevent and control (*Abdelrhim et al., 2018; Cowger et al., 2018*). Using cultivars carrying major resistance genes (known as ~~a~~ *Pm* genes) is the most economical and cost-effective way to control wheat powdery mildew (*Lu et al., 2020; Parks et al., 2008*). To date, more than 91 *Pm* resistance genes, mapped to 58 loci, have been identified that confer resistance to wheat powdery mildew (*Nanjundan et al., 2020; Petersen et al., 2015; Tan et al., 2019; Xu et al., 2020*). However, resistant cultivars carrying *Pm* genes confer complete resistance to specific *Bgt* races leading to high pressure on fungus. Due to genetic variations ~~occurs~~ occurring in fungal races, wheat cultivars carrying effective resistance genes are not able to recognize *Bgt* races anymore and turn in~~-~~to susceptible cultivars (*Wolfe and Schwarzbach 1978; Wu et al., 2019*). Monitoring of population dynamic and genetic variation analysis of *Bgt* provide a basis for timely warning and sustainable management of wheat powdery mildew using disease-resistant cultivars.

In China, wheat powdery mildew disease mainly occurs in the Yunnan-Guizhou-Sichuan zone, the wheat region in the middle and lower reaches of

the Yangtze River, and the wheat region in Huang-Huai-Hai Region and Ningxia, Inner Mongolia, and northeast China (*Sheng et al., 1995*). The spread of wheat powdery mildew in China also occurs from south to north over long distances in spring, and in the opposite direction in autumn (*Wu et al., 2019*). Northeast China has acold winters that last more than 6 months, therefore the local wheat powdery mildew *Bgt* does not survive as a primary inoculum for the following year. Apparently, the initial inoculum for the spring season of Northeast China originates from the south China, the Jiaodong Peninsula region (*Yang et al., 1992*). The data of inter simple sequence repeat (ISSR) analysis confirmed that Shandong, Henan, Hubei, and especially Shandong provided the initial inoculum of *Bgt* for the spring wheat area of Northeast China (*Zhu et al., 2015*).

Assessment of infection type on wheat differential lines is the most basic method to identify the races of *Bgt* and to analyze virulence genes and virulence evolution of pathogenic populations (*Imani et al., 2002; Liu et al., 2015; Niewoehner et al., 1998*). However, with the development of molecular biology techniques, DNA molecular markers has have been used extensively in the analysis of *Bgt* population evolution (*Comlekcioglu et al., 2010; Shao et al., 2011; Zhu et al., 2010*). Our previous analysis revealed that isolates collected in 2013 and 2014 from the northeast and northwest China (Gansu, Heilongjiang, and Liaoning), have a clear genetic relationship (*Wu et al., 2019*). In this study, the genetic diversity of these *Bgt* isolates were was analyzed using Expressed sequence tag-simple sequence repeat (EST-SSR) molecular markers to explore the genetic structure of the *Bgt* population and the relationship between different populations in the two regions. The data were obtained from genetic structure and the distribution of *Bgt* isolates will be discussed.

Materials and Methods

Collection of *Bgt* isolates. From May to July 2015, diseased leaves with fresh powdery mildew spores were collected from Liaoning and Heilongjiang and kept in falcon tubes containing water agar medium with 1% of 6-benzylaminopurine preservation solution (40 mg·L^{-1}). Diseased leaves carrying the cleistothecium of *Bgt* were also collected from Sichuan, brought back to the laboratory, dried in a cool place, and stored at 4 °C. All isolates used in the study were identified by XXW, WLW, and TYL, and all isolates were deposited (no deposition number) in the College of Plant Protection, Shenyang Agricultural University (our lab).

Isolation, purification, and propagation of *Bgt.* The highly susceptible Little Club (provided by the College of Plant Protection of Shenyang Agricultural University) ~~were~~ was sowed in tile pots. When the seedlings had grown to the one leaf stage, 5-6 cm leaf segments were cut and placed in a petri dish lined with double filter paper. Five to ~~six~~ six-leaf segments were placed face up in each petri dish and held in place with glass strips on both ends. The filter paper was moistened with 40 mg·L^{-1} of 6-benzylaminopurine (6-BA) preservation solution. The collected powdery mildew was first attached to the front of the leaf with a toothpick sharpened into a flattened tip. Three spots were attached to each leaf, lightly and evenly applied taking care not to scratch the leaf. After inoculation, the leaves were incubated for 5-7 days in a growth chamber at 18-22°C with a ~~14~~ 14-hour/10-hour light/dark cycle. After white spore mounds appeared on the leaves, the leaves were inoculated by shaking. The spores on one leaf segment were gently shaken off on the freshly isolated leaf segment. After incubation at 18-22℃ for 5-7 days under light, single colonies were inoculated at 3 points on the isolated leaf segments with a flat toothpick, and this process was repeated several times for the isolation and purification of monospores. The isolated and purified single pustules were numbered and multiplied for preservation

Release of *Bgt* cleistothecium ascospores. A cleistothecium was picked from diseased leaves using an inoculating needle and incubated on moistened filter paper soaked with distilled water. After 5 days, the cleistothecium was picked and observed under a microscope. When ascospores were formed, a cleistothecium was randomly picked from diseased leaves and transferred to moistened filter paper sheets, with one cleistothecium per sheet. The filter paper sheet was then placed upside down in the center of a petri dish lid, the petri dish lid was placed on the petri dish with the isolated wheat leaves, and the petri dish was sealed with parafilm and placed in a light incubator at 17°C.

Analysis of virulence frequency of *Bgt.* A total of 36 single-gene (*Pm*) lines (provided by the College of Plant Protection of Shenyang Agricultural University) were sown in 10 cm diameter tile pots according to their numbers and marked. The highly susceptible variety Chancellor was used as the susceptible control. The first leaf was cut when the seedlings reached the one leaf stage and the cut leaf segments were placed in order in the Petri dishes, and the propagated single pustules were gently shaken off the leaves. The culture was incubated for 5-7 days. When the susceptible control was fully developed, the infection types (ITs) were assessed and recorded following the method described by the previous study (*Si et al., 1987*): ITs 0-2 were marked as resistant (R) and the corresponding isolates as avirulent; ITs 3-4 were marked as susceptible (S) and the corresponding isolates as virulent. The test was repeated 3 times.

Genomic DNA extraction and polymerase chain reaction (PCR) analysis. Genomic DNA was extracted from conidia using the Omega Bio-Tek fungal DNA kit (Norcross, GA, USA) following the manufacturer's protocol. The total PCR reaction volume was 20 μL, consisting of 1 μL DNA template (30 ng·μL^{-1}), 1 μL forward

primer, 1 μL reverse primer, 10 μL 2 × Power *Taq* PCR Master Mix, and 7 μL ddH_2O. The PCR procedure was as follows: initial denaturation at 94℃ for 5 min; then 35 cycles of a denaturation step at 94℃ for 30 s and an extension step at 72℃ for 1.5 min, followed by a final extension at 72℃ for 10 min.

Selection of the EST-SSR primers. Seven pairs of EST-SSR primers (Table 1) were designed according to Xu (2012) and were screened for the occurrence of clear and stable polymorphisms. Two pairs were chosen for the genetic polymorphism analysis. The primers were synthesized by Sangon Biotech Inc. (http://www.sangon.com/, Shanghai, China).

Polyacrylamide gel electrophoresis and genetic diversity analysis. The procedure for polyacrylamide gel electrophoresis (PAGE) was as previously published (*Chen et al., 2015*). The silver staining method was used to visualize the PCR products as described by Bassam *et al.* (1991). Based on the PAGE results, '1' or '0' were assigned to the presence or absence of bands, respectively, and the same method was used for frequency of virulence analysis, '1' or '0' were assigned to resistance (ITs: 0-2) or susceptibility (ITs: 3-4), respectively, in the host. According to the '1, 0' data matrix, the genetic similarity was calculated using NTSYSpc 2.10 statistical software. The unweighted pair group arithmetic method was used for gene diversity cluster analysis of the expression sequences and then the classification trees were constructed.

Results

Virulence frequencies of 80 isolates to 36 single gene lines. A total of 80 *Bgt* isolates, collected from Liaoning, Heilongjiang, and Sichuan, were isolated. Virulence frequencies of these isolates were assessed on 36 differential lines individually

containing single powdery mildew (*Pm*) resistance gene. The results show that the virulence frequency of isolates from northeast China against resistance genes including *Pm1*, *Pm2*, *Pm3c*, *Pm3d*, *Pm3e*, *Pm3f*, *Pm4a*, *Pm4b*, *Pm5*, *Pm6* (Coker747), *Pm6* (Tingalen), *Pm7*, *Pm8*, *Pm16*, *Pm17*, *Pm19*, *Pm23*, *Pm24*, *Pm30*, *Pm4+8*, *Pm4b+mli*, *Pm4+2X*, *Pm1+2+9*, and *Era* was above 60%, indicating that the effectiveness of these resistance genes had been partially or completely lost. Virulence to *Pm 3a*, *Pm3b*, *Pm13*, *Pm18* (*1c*), *Pm20*, *Pm22* (*1e*), *Pm5+6*, *Pm2+mld* , and *PmV2+6* was 40%-60%. Virulence to *PmVXBD* and *Pm21* were 27.5% and 0 (Table 2).

EST-SSR analysis of *Bgt* isolates. Two pairs of EST-SSR namely Blu SSR3-1-Blu SSR3-2 and Blu SSR29-1-Blu SSR29-2, which amplified clear and stable polymorphic bands, were chosen from among 7 reported EST-SSR primer pairs. These two pairs of primers were specific to *Bgt* isolated from Northeastern China and Gansu Province. Fig. 1 and 2 show the PAGE results of EST-SSR polymorphism using primer pairs Blu SSR3-1- plus Blu SSR3-2 and Blu SSR32-1- plus Blu SSR32-2, respectively. Genetic similarity analysis of EST-SSR PAGE, was assessed on 80 *Bgt* isolates collected from Liaoning, Heilongjiang, and Sichuan using NTSYSpc 2.10. When the genetic similarity coefficient was 0.782, excluding H18, H38, and H38 from Heilongjiang and L34 and L44 from Liaoning, the remaining 62 isolates were divided into eight groups: Group A consisted of 18 isolates, including ten isolates from Liaoning and eight isolates from Heilongjiang (Fig. 3); Group B consisted of four isolates, including two isolates from Heilongjiang and two isolates from Liaoning; Group C consisted of six isolates, including three isolates from Heilongjiang, two isolates from Sichuan and one isolate from Liaoning; Group D consisted of three isolates from Liaoning; Group E consisted of 13 isolates, including

12 isolates from Sichuan and one isolate from Heilongjiang; Group F consisted of two isolates, including one isolate from Sichuan and one isolate from Liaoning; Group G consisted of 13 isolates, including 11 isolates from Sichuan, two isolates from Heilongjiang; Group H consisted of two isolates from Liaoning. Overall, there was a certain degree of transmission among *Bgt* isolates in different regions. However, when the genetic similarity coefficient was high, some isolates from Heilongjiang and Liaoning were clustered into subcategories, while isolates from Sichuan were clustered into subcategories separately, indicating that the genetic exchange between isolates from Heilongjiang and Liaoning was extensive. At the same time, it showed that there were genetic differences among these *Bgt* groups from different regions.

Virulence diversity and genetic diversity of *Bgt.* According to the results obtained from EST-SSR polymorphism of 36 isolates (12 isolates were randomly selected from each of the three provinces), a genetic evolution tree was constructed with NTSYSpc 2.10 as shown in Fig. 4. With a genetic similarity coefficient of 0.77, excluding L33 and L30 from Liaoning, 34 out of 36 isolates were divided into six groups: Group A, consisted of three isolates, including two isolates from Liaoning and one isolate from Heilongjiang; Group B~~,~~ consisted of five isolates including three isolates from Sichuan, one isolate from Liaoning and one isolate from Heilongjiang; Group C, consisted of nine isolates, including eight isolates from Sichuan and one isolate from Heilongjiang; Group D, consisted of 11 isolates, including five isolates from Heilongjiang and six isolates from Liaoning; Group E, consisted of three isolates from Heilongjiang; Group F, consisted of three isolates from Liaoning, Heilongjiang, and Sichuan, respectively.

The 36 isolates were divided into different sub-categories according to the region when the genetic similarity was high. At a genetic similarity coefficient of 0.842,

group C could be divided into three subgroups: Subgroups I and II consisted of isolates from Sichuan, while Subgroup III consisted of two isolates from Sichuan (C17, C6-2) and one isolate from Heilongjiang (H19). At a genetic similarity coefficient of 0.833, Group D could be divided into three subgroups: Subgroup I, consisted of three isolates from Liaoning (L23, L60, L17); Subgroup II, consisted of two isolates from Heilongjiang (H1, H16) and one isolate from Liaoning (L38); Subgroup III, consisted of three isolates from Heilongjiang (H46, H7, H40) and two isolates from Liaoning (L39, L50).

The virulence diversity "0, 1" matrix of these isolates was constructed based on the infection type of 36 *Bgt* isolates on 34 identified hosts. The phylogenetic tree clustered according to the similarity of infecting hosts is shown in Fig. 5. When the genetic similarity coefficient was 0.677, the 36 isolates were divided into two groups: Group A consisted of nine isolates from Heilongjiang (Fig. 5). When the genetic similarity coefficient increased to 0.787, except for H31 and C37 from Heilongjiang and C13-5 from Sichuan, 33 out of 36 isolates were divided into six groups: Group A consisted of 19 isolates, including 10 isolates from Sichuan, seven isolates from Liaoning (L30, L17, L61, L39, L1, L23, L33) and two isolates from Heilongjiang (H19, H9); Group B consisted of six isolates, including five isolates from Liaoning (L38, L60, L50, L42, L48) and one isolate from Heilongjiang (H16); Groups C, D, E and F each consisted of two isolates from Heilongjiang.

The EST-SSR polymorphism and virulence diversity dendrograms of these isolated genes revealed that when the similarity coefficient was > 0.77, there were four clusters of genetic and virulence diversity: Group A included C36 and L1; Group B included H40 and H1; Group C included L38, L60, L50, and H16; Group D included C6-1, C2-3, C35, C14, C13-3, C5-1, C17, C6-2, and H19. The positions of other

isolates in the genetic polymorphism and virulence diversity dendrograms were different and did not correspond to each other. Thus, there was a correlation between genetic polymorphism and virulence diversity of 18 isolates (50%). However, the EST-SSR polymorphism of isolated genes did not correspond to the virulence diversity of isolates in the single-gene lineage identification of hosts.

Discussion

Due to the gene-for-gene relationship between wheat and its fungal pathogen *Bgt*, studies of physiological race play an impressive role in monitoring the population dynamics of fungus. Continuous studies shows that the virulence of *Bgt* is increasing year by year and cultivars carrying resistance genes tend to lose their effectiveness. In 2008 - 2009 the resistance genes *Pm4b*, *Pm2+6*, *Pm4+8*, *Pm12*, *Pm16*, *Pm18* (*Pm1c*), *Pm20*, *Pm21*, *Pm22* (*Pm1e*), and *Pm23* were effective against isolates from northeast China (*Chi 2019*). In 2011-2012, the resistance genes *Pm2*, *Pm4a*, *Pm4b*, *Pm12*, *Pm13*, *Pm16*, *Pm18* (*Pm1c*), *Pm19*, *Pm20*, *Pm21*, *Pm22* (*Pm1e*), *Pm23*, *and Pm5+6* were effective against isolates from northeast China (*Chen et al., 2013*). In 2013-2014 the resistance genes *Pm13*, *Pm16*, *Pm18* (*Pm1c*), *Pm21*, *Pm22* (*Pm1e*), and *PmXBD* were effective against isolates from northeast China. In the present study, we found that only resistance genes *Pm21* and *PmXBD* are effective against *Bgt* isolates collected in 2015 from northeast China. Therefore, continuous virulence monitoring of *Bgt* can provide a reliable basis for breeding for disease resistance in Northeast China. Although the incidence rates of the virulence genes *V18 (V1c)* and *V22 (V1e)* increased to between 40% and 50%, their corresponding resistance genes still have a moderate value. Additionally, only *Pm21* constitutes an effective resistance gene towards isolates from Sichuan. The resistance genes *Pm18 (Pm1c)* and *Pm22 (Pm1e)* are of average resistance but can still be used. Gene *Pm21* is transferred from

Haynaldia villosa, it has been widely used in a wheat breeding program in China (*Cao et al., 2011; Wu et al., 2019*). According to statistics, more than 10 commercial wheat have been released since 2002, with a planting area of more than 3.4×10^6 hm^2 (*Cao et al., 2011*). This gene ~~encoding~~ encodes a typical CC-NBS-LRR (NLR for short) protein which recognises the presence of specific pathogen 'avirulence' molecules and thus induces host ~~defences~~ defenses (*He et al., 2018*). As NLRs are recognition proteins, the resistance they control is almost always readily overcome by mutations in the pathogen's avirulence protein which prevent it from being ~~recognised~~recognized, so host ~~defences~~ defenses aren't induced. For example, a few researchers reported isolates with virulence to the gene, which should be paid great attention (*Li et al., 2016*).

According to the occurrence of *Bgt* virulent isolates among different provinces, there is a regional difference between the distribution of virulence genes and the incidence rate. Results show that the incidence rates of *V13* in Heilongjiang and Liaoning were 18.5% and 62.1%, respectively, the incidence rate of *V2+mld* in Heilongjiang and Liaoning was 22.2% and 79.3%, respectively, the incidence rate of *V2* in Heilongjiang and Liaoning in 2015 was 33.3% and 93.1%, ~~respectively,~~ and the incidence rate of *V8* in Heilongjiang and Liaoning was 33.3% and 86.2%, respectively. This indicates that *Pm2*, *Pm8*, *Pm13*, and *Pm2+mld* are effective resistance genes in Heilongjiang but are ineffective when used alone in Liaoning. Similarly, the incidence rate of the virulence genes corresponding to the resistance genes *Pm4b+mli* and *Era* is much higher in Liaoning than in Heilongjiang. These resistance genes have almost completely lost their effectiveness in Liaoning, but still have some effectiveness in Heilongjiang, and they still show clear superiority in the disease resistance breeding process of isolates from Heilongjiang.

Genetic diversity, which is also known as genetic polymorphism, is of great significance to species adapting to environmental changes and also for survival and replication. With the development of molecular biology, molecular marker technology has been widely used in the genetic diversity analysis of plant pathogenic bacteria. Molecular marker technology was used to study the genetic diversity of *Bgt*, to explore its genetic variation and regional transmission, providing a prerequisite basis for the effective prevention and control of wheat powdery mildew and the rational distribution of disease-resistant cultivars (*Jia et al., 2008; Wang et al., 2001; Wolfe and Schwarzbach 1978*). In this experiment, the genetic diversity of *Bgt* isolates from northeast China and Sichuan was explored from the perspective of gene expression sequences using EST-SSR molecular marker technology. Genetic differences in the expressed sequences of *Bgt* from different regions in 2015 were analyzed. Virulence diversity and genetic diversity were also compared. The results showed that EST-SSR molecular marker technology appropriately revealed the genetic diversity of *Bgt*.

Based on the similarity coefficient, cluster analysis of *Bgt* isolates revealed that some isolates collected from Liaoning, Heilongjiang, and Sichuan in 2015 were clustered together, indicating that these isolates might have a certain degree of transmission and exchange, with more exchange rate for Liaoning and Heilongjiang isolates. However, at a higher genetic similarity coefficient, isolates from different regions were clustered into small groups, indicating larger genetic differences among them.

When comparing the genetic diversity and host virulence polymorphism of *Bgt* with the virulence polymorphism on the host, we found that 18 out of 36 sample isolates were clustered together in genetic diversity and virulence diversity clusters in four different combinations. To some extent, the EST-SSR molecular marker

technology revealed a correlation between *Bgt* genetic diversity and virulence diversity. However, the genetic diversity and virulence diversity of 50% of the isolates were different and did not correspond to each other.

Acknowledgments

This study was supported by the Natural Science Foundation of Education Department of Liaoning Province (No. LJKZ0648), the Natural Science Foundation of Liaoning Province (2020-MS-204).

Declaration of Competing interests

The authors declare that they have no competing interests.

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