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Molecular Identification of Wheat Leaf Rust Resistance Genes in Sixty Chinese Wheat Cultivars

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Abstract

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Common wheat (*Triticum aestivum* L.) is the major crop cultivated in Xinjiang and Anhui provinces of China. The climate in these two provinces is favourable for wheat leaf rust (*Puccinia triticina*) (*Pt*) infection. Here, we demonstrate a detailed investigation on the leaf rust resistance of 60 major wheat cultivars cultivated in these two regions. A mixture of high virulent *Pt* races (THTT, THTS, THTQ and PHPS) were used to phenotype all the collected wheat cultivars at an adult plant stage. Phenotypic disease severity (FDS) and the area under the disease progress curve (AUDPC) for each of these wheat cultivars was calculated. Among all the tested wheat cultivars, three cultivars (Xindong20, Xindong 29 and 99AR142-1) with the lowest FDS and AUDPC may carry major resistance genes. Twenty-seven cultivars (45% of the total tested ones) showed a relatively lower resistance with an average of 12.52% FDS and 126.3 AUDPC. Minor resistance or slow rusting genes may be present in this group of cultivars. Molecular markers for the leaf rust resistance genes *Lr1*, *Lr9*, *Lr19*, *Lr24*, *Lr26* and *Lr34* were further used for genotypic screening. *Lr1*, *Lr19*, *Lr26* and *Lr34* were detected in 19 (31.7%), 1 (1.7%), 12 (20%) and 6 (10%) wheat cultivars, respectively. Neither *Lr9* nor *Lr24* could be detected in any of the tested cultivars. These results will greatly improve wheat molecular breeding for leaf rust resistance in these areas.

Keywords: molecular-assisted selection; *Puccinia triticina*; STS markers; *Triticum aestivum* L.

Wheat leaf rust, caused by *Puccinia triticina* (*Pt*), is one of the most severe fungal diseases on wheat leaves. Its infection seriously disrupts plant photosynthesis and results in up to 40% of yield losses under favourable conditions (Knott 1989). Wheat leaf rust is a global fungal disease that can spread rapidly by wind (Huerta-Espino *et al.* 2011). In the major wheat cultivation area of Northern China, there were several destructive epidemics of wheat leaf rust in the 1970s (Dong 2001) and occasional severe yield losses during the past decades (Zhao *et al.* 2008). With the trends of global warming, climates in some parts of this region (*e.g.* Xinjiang, Anhui, Gansu, Sichuan, Shaanxi and Henan provinces) become more favourable for wheat leaf rust infection (Zhou *et al.* 2013).

Utilization of resistant cultivars is still the most efficient, economic, and eco-friendly way to control leaf rust (Line & Chen 1995). A large number of wheat cultivars in China, both local farming cultivars and introduced exotic cultivars, showed a moderate or higher leaf rust resistance. An urgent need is to clarify the profiles of leaf rust resistance genes in these cultivars, since many of them have lost their resistance to newly emerged virulent leaf rust races in China. In the early 1990s, the distribution of leaf rust resistance genes *Lr9*, *Lr19*, *Lr24* and *Lr34* in

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major cultivated wheat cultivars was investigated. These cultivars provide good sources for developing leaf rust resistance during wheat breeding programs (Li & Shang 2005).

In recent years, molecular markers have been widely used for various applications in plant genotyping. Simple sequence repeat (SSR) marker, which is composed of a series of conserved nucleotides, is a type of molecular marker with advantages of higher repeatability, lower DNA usage and identification of heterozygosity and homozygosity (Moore *et al.* 1991). Another molecular marker, sequence tagged site (STS) marker, was designed for specific single-copy DNA fragments in the whole genome. STS markers for wheat leaf rust resistance genes *Lr1* (Cloutier *et al.* 2007), *Lr*9 (Schachermayr *et al.* 1994), *Lr19* (Gupta *et al.* 2006a), *Lr24* (Gupta *et al.* 2006b), *Lr26* (Froidmont 1998) and *Lr34* (Dyck 1987) were used in this study. This study was aimed to identify the leaf rust resistance genes in 60 wheat cultivars from Anhui and Xinjiang provinces in China.

MATERIAL AND METHODS

Plant materials and *Puccinia triticina* (*Pt*) isolates. Sixty wheat cultivars were collected from major cultivated ones in Anhui and Xinjiang provinces of China. Four virulent *Pt* races THTT, THTS, THTQ and PHPS were annotated using a Prt-coding system (Long & Kolmer 1989) and then all the 60 cultivars were inoculated with them at adult stages in the field (Hebei Agricultural University, Baoding, China, 2014–2015). These leaf rust races were preserved at Biological Control Centre for Plant Diseases and Plant Pests of Hebei, Hebei Agricultural University, China.

Field trials were conducted in randomized complete blocks with two replications. Each plot consisted of a single 1.5 m row with 30 cm row distance. About 100 seeds for each cultivar were sown in each row. Every tenth row was sown with the susceptible line Zhengzhou5389 as a control to facilitate rust development. Equal amounts of *Pt* races THTT, THTS, THTQ and PHPS were mixed with a few drops of Tween 20 (0.03%) and sprayed to control rows at the jointing growth stage at dusk. The average temperature in the middle of April at Baoding was about 15°C during the day and 10°C during the night. After inoculation, plants were immediately covered with plastic films to keep moisture within plant canopies and the films were removed next morning. The experimental field was irrigated after inoculation.

Leaf rust resistance assessment. Assessment of leaf rust resistance was initiated when the susceptible control Zhengzhou5389 had more than 50% disease severity (the rust incidence area accounted for more than half of the whole leaf) and disease scoring was carried out once a week until the disease severity of Zhengzhou5389 reached 100% (Peterson *et al.* 1948). Assessment of the final phenotypic disease severity (FDS) data was used for gene identification. The area under the disease progress curve (AUDPC) is a way to analyse the disease severity data (Broers *et al.* 1996). AUDPC was calculated as:

$$\text{AUDPC} = \sum_{i=1}^{n} [(X_{i+1} + X_i)/2][T_{i+1} - T_i]$$

where:

T_i – number of days after inoculation

X_i – disease severity

DNA extraction and molecular marker screening. Genomic DNA was extracted from the seedlings of the tested wheat cultivars using the CTAB method (Sharp *et al.* 1988). The DNA was quantified with a UV spectrophotometer, and diluted to a final concentration of 30 ng/µl.

The PCR reactions were applied in volumes of 10 µl with 1.0 U Taq of DNA polymerase (Zexing Biotechnology Co., Ltd., Beijing, China), 1× PCR buffer (Zexing Biotechnology Co., Ltd.), 100 µM each of dNTP (Sangon Biotech Co., Ltd., Shanghai, China), 3 pmol each primer and 30 ng of template DNA. The PCR conditions were denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55–60°C (depending on the primer pair) for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was mixed with 2 µl of formamide loading buffer (98% formamide, 10 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, pH 8.0). The mixture was then loaded on 10% non-denaturing polyacrylamide gels or 1.5% agarose gel for electrophoresis.

RESULTS

Field identification reactions. The 60 cultivars were inoculated with the mixture of the *Pt* races THTT, THTS, THTQ and PHPS in the field. FDS and AUDPC for each of the cultivars were calculated (Table1). Among all the 60 tested cultivars, three cultivars (5% of all the tested cultivars) with average FDS 3.67% and AUDPC 42.0 showed high resistance to leaf rust. We speculate these three cultivars may

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Table 1. Molecular validation of leaf rust resistance genes and resistant phenotypes at an adult stage in 60 wheat cultivars

No	Cultivars	Source	FDS (%)	AUDPC	Resistance genes				
					Lr9	*Lr19*	*Lr24*	*Lr26*	*Lr34*
1	Xindong33	Xinjiang	15	126	–	–	–	–	–
2	Xindong17	Xinjiang	25	175	–	–	–	–	–
3	Xindong18	Xinjiang	30	297.5	–	–	–	–	–
4	Xindong20	Xinjiang	5	56	–	–	–	–	–
5	Xindong22	Xinjiang	25	231	–	–	–	–	–
6	Xindong32	Xinjiang	20	192.5	–	–	–	–	–
7	Xindong38(0138)	Xinjiang	17.5	183.8	–	–	–	–	–
8	Kendong00(2)	Xinjiang	30	280	–	–	–	–	–
9	Kendong03(77)	Xinjiang	15	122	–	–	–	–	–
10	2005(56)/6-2-2	Xinjiang	10	126	–	–	–	–	–
11	2005(65)/7-2-1	Xinjiang	15	91	–	–	–	–	–
12	2006(63)/7-3-2	Xinjiang	17.5	236.3	–	–	–	–	–
13	2006(114)/5-10-1	Xinjiang	50	280	–	–	–	–	–
14	2006 LunI/11-1-3	Xinjiang	17.5	148.8	–	–	–	–	–
15	2007LunII//6-3-1	Xinjiang	40	280	–	–	–	–	–
16	2007(32)/7-4-3	Xinjiang	20	157.5	–	–	–	–	–
17	2007(96)/15-1-1	Xinjiang	30	227.5	–	–	–	–	–
18	2007(96)/15-3-7	Xinjiang	15	140	–	–	–	–	–
19	2007(96)/21-1-1	Xinjiang	25	262.5	–	–	–	–	–
20	Xindong 38(CA9719-9)	Xinjiang	30	297.5	–	–	–	–	–
21	Han 5316	Xinjiang	37.5	218.5	–	–	–	+	–
22	Han 6172	Xinjiang	25	175	–	–	–	–	–
23	Shidong 7	Xinjiang	17.5	99.8	–	–	–	–	–
24	Shidong 8	Xinjiang	15	91	–	–	–	–	–
25	Shidong 9	Xinjiang	30	143.5	–	–	–	–	–
26	Yi'nong 16	Xinjiang	7.5	36.8	–	–	–	–	–
27	Yi'nong 18	Xinjiang	27.5	183.8	–	–	–	+	+
28	Xindong 2	Xinjiang	40	385	–	–	–	–	–
29	Xindong 14	Xinjiang	50	367.5	–	–	–	–	+
30	Xindong 15	Xinjiang	27.5	169.8	–	–	–	+	+
31	Xindong 16	Xinjiang	30	178.5	–	–	–	–	–
32	Xindong 17	Xinjiang	25	175	–	–	–	–	–
33	Xindong 21	Xinjiang	25	175	–	–	–	–	–
34	Xindong 23	Xinjiang	37.5	239.8	–	–	–	+	–
35	Xindong 24	Xinjiang	17.5	113.8	–	–	–	–	–
36	Xindong 27	Xinjiang	10	122.5	–	–	–	–	–
37	Xindong 28	Xinjiang	25	161	–	–	–	–	–
38	Xindong 29	Xinjiang	5	56	–	–	–	–	+
39	Xindong 30	Xinjiang	25	231	–	–	–	–	–
40	Xindong 31	Xinjiang	45	350	–	–	–	+	–
41	Kuidong 4	Xinjiang	15	91	–	–	–	+	–
42	Kuihua 1	Xinjiang	17.5	166.3	–	–	–	–	–
43	03-6118	Xinjiang	32.5	271.5	–	–	–	–	–

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Table 1 to be continued

No	Cultivars	Source	FDS	AUDPC	Resistance genes				
					Lr9	*Lr19*	*Lr24*	*Lr26*	*Lr34*
44	99-5091	Xinjiang	50	385	–	–	–	–	–
45	95(20)/8-1-2	Xinjiang	40	437.5	–	–	–	–	–
46	99AR142-1	Xinjiang	1	14	–	–	–	–	–
47	99AR144-1	Xinjiang	8	66.5	–	–	–	+	+
48	99(79)/2-1-2	Xinjiang	10	108.5	–	–	–	+	+
49	99(55)/3-1-1	Xinjiang	12.5	131.3	–	–	–	–	–
50	99(36)/2-2-2	Xinjiang	40	297.5	–	–	–	–	–
51	12P103 Wanmai 53	Anhui	17	112	–	–	–	–	–
52	12P101 Wanmai 50	Anhui	17.5	183.8	–	–	–	–	–
53	12P124 Wanmai 68	Anhui	20	108.5	–	–	–	+	–
54	12P123 Wanmai 46	Anhui	15	126	–	–	–	+	–
55	12P119 Fu 936	Anhui	25	175	–	–	–	+	–
56	Wanmai 47	Anhui	10	73.5	–	–	–	–	–
57	12P106 Su 553	Anhui	35	455	–	+	–	–	–
58	12P114 Annong 0305	Anhui	30	227.5	–	–	–	+	–
59	12P122 Wanmai 38	Anhui	10	122.5	–	–	–	–	–
60	12P120 An 1331	Anhui	12.5	131.3	–	–	–	–	–

FDS – phenotypic disease severity; AUDPC – area under the disease progress curve; resistance genes: + the fragment is amplified; – no specific fragment is amplified

carry major resistance genes. Twenty-seven cultivars (45%) with average FDS 12.52% and AUDPC 126.3 showed a relatively lower resistance to leaf rust. Minor resistance or slow rusting genes may function in these cultivars. Twenty-six cultivars (43.3%) with average FDS 30.48% and AUDPC 244.3 showed moderate resistance. Resistance genes in these cultivars might be overcome by some of the applied virulent *Pt* races.

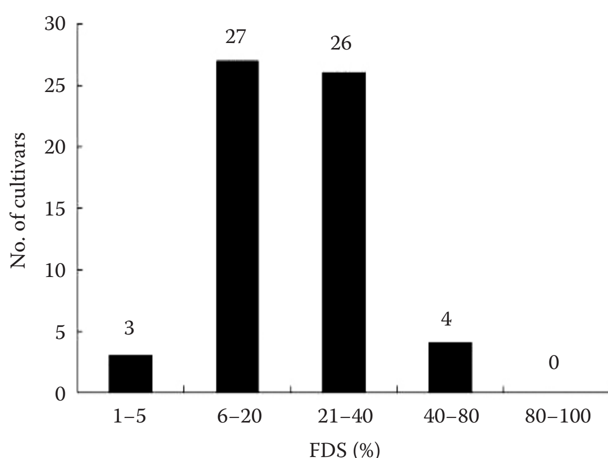

Figure 1. Distribution of the phenotypic disease severity (FDS) in 60 wheat cultivars

Four cultivars (6.7%) with average FDS 48.75% and AUDPC 345.6 showed a susceptible phenotype to inoculated *Pt* races (Table 1 and Figure 1).

Molecular marker analysis. The STS marker *WR003* is closely linked with the leaf rust resistance gene *Lr1* (Table 2). Using this marker, extracted genomic DNA of all the tested cultivars was screened and their PCR products were analysed by agarose gel electrophoresis. Nineteen cultivars (31.67% of all the tested cultivars) showed the same band (760 bp) as near-isogenic lines (NILs) carrying *Lr1* (Figure 2). The average FDS and AUDPC for these 19 cultivars were 19.8% and 160.6, respectively.

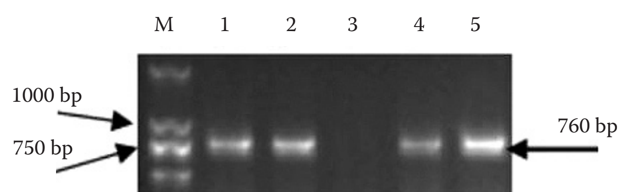

Figure 2. Amplification of specific PCR fragments of the STS marker *WR003*

M – DL2000 marker; 1 – TcLr1; 2 – Shidong 8; 3 – Shidong 9; 4 – Yi'nong 16; 5 – Yi'nong 18

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Table 2. STS and SCAR markers and their conditions for detecting leaf rust resistance (*Lr*) genes as well as annealing temperatures

Lr gene	Marker type	Primer	Sequence of primer (5'–3')	Annealing temp. (°C)	Size (bp)	Reference
Lr1	STS	WR003-F WR003-R	GGGACAGAGACCTTGGTGGA GAC GATGATGATTTGCTGCTGG	65	760	Qiu *et al.* (2007)
Lr9	STS	J13/1 J13/2	TCCTTTTATTCCGCACGCCGG CCACACTACCCCAAAGAGAG	66	1100	Gupta *et al.* (2005)
Lr9	STS	SCS5-550F SCS5-550R	TGCGCCTTCAAAGGAAG TGCGCCCTTCTGAACTGTAT	60	512	Gupta *et al.* (2005)
Lr19	SCAR	SCS265-F SCS265-R	GGCGGATAAGCAGAGCAGAG GGCGGATAAGTGGGTTATGG	65	737	Gupta *et al.* (2006a)
Lr24	STS	J09/1 J09/2	TCTAGTCTGTACATGGGGGC TGGCACATGAACTCCATACG	60	310	Schachermayr *et al.* (1995)
Lr26	STS	Glu-B3F Glu-B3R	GGTACCAACAACAACAACCC GTTGCTGCTGAGGTTGGTTC	65	210	Froidmont (1998)
Lr26	STS	ω-secalin F ω-secalin R	ACC TTCCTCATCTTTGTCCT CCGATGCCTATACCACTACT	65	1076	Chai *et al.* (2006)
Lr34	STS	csLV34 F csLV34 R	GTTGGTTAAGACTGGTGATGG TGCTTGCTATTGCTGAATAGT	58	150	Lagudah *et al.* (2006)

The STS marker *SCS265* is closely linked with the leaf rust resistance gene *Lr19* (Gupta *et al.* 2005). A similar screening process was carried out. Only one cultivar (1.7%) showed the same band (737 bp) as NILs carrying *Lr19* (Table 1 and Figure 3). The FDS and AUDPC for this line were 40% and 472.5, respectively.

Lr9 and *Lr24* are closely linked with specific STS markers *J13* and *OPJ-09*, respectively. No cultivars could be detected carrying either of these two genes (Figure 4 and Figure 5).

Wheat cultivars carrying *Lr26* can be validated by the *ω-secalin* marker with a band of 1076 bp (Figure 6).

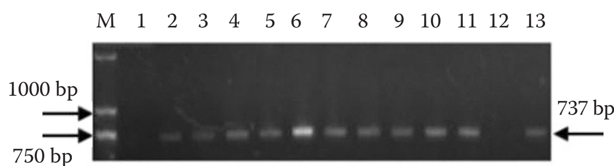

Figure 3. Amplification of specific PCR fragments of the STS marker *Lr19-SCS265*

M – DL2000 marker; 1 – TcLr19; 2 – Shidong 8; 3 – Shidong 9; 4 – Yinong 16; 5 – Yinong 18; 6 – Xindong 2; 7 – Xindong 14; 8 – Xindong 15; 9 –Xindong 14; 10 – Xindong 15; 11 – Xindong 16; 12 – 12P106Su 553; 13 – Xindong 17

The *Glu-B3* marker with product size of 636 bp can be used for the validation of none-*Lr26* cultivars (Figure 7). During our screening, 12 cultivars (20.0%)

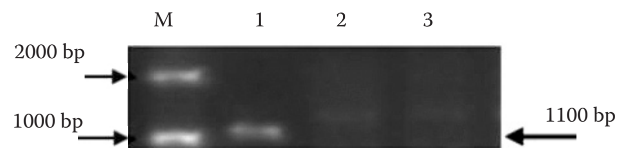

Figure 4. Amplification of specific PCR fragments of the STS marker *Lr9-J13*

M – DL2000 marker; 1 – TcLr9; 2 – Shidong 8; 3 – Shidong 9

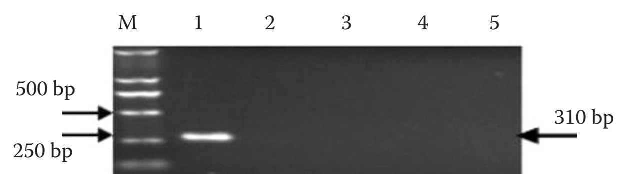

Figure 5. Amplification of specific PCR fragments of the STS marker *Lr24-OPJ-09*

M – DL2000 marker; 1 – TcLr24; 2 – Shidong 8; 3 – Shidong 9; 4 – Yinong 16; 5 – Yinong 18

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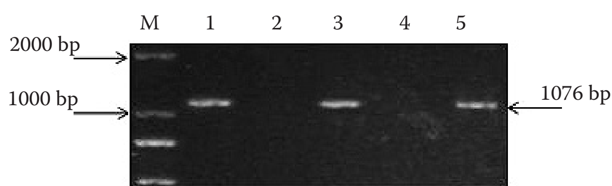

Figure 6. Amplification of specific PCR fragments of the STS marker *Lr26-ω-secalin*
M – DL2000 marker; 1 – TcLr26; 2 – Shidong 8; 3 – Xindong 15; 4 – Yinong 16; 5 – Yinong 18

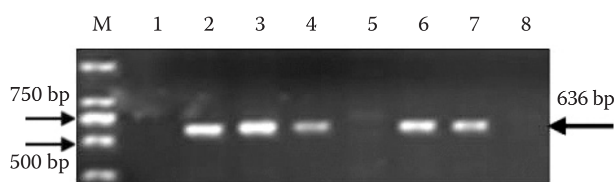

Figure 7. Amplification of specific PCR fragments of the STS marker *Lr26-Glu-B3*
M – DL2000 marker; 1 – TcLr26; 2 – Shidong 8; 3 – Shidong 9; 4 – Yinong 16; 5 – Yinong 18; 6 – Xindong 2; 7 – Xindong 14; 8 – Xindong 15

were speculated as carrying *Lr26* with average FDS 24.2% and AUDPC 172.

The STS marker *csLv34* was used for the screening of *Lr34* in the collected cultivars. Six cultivars (10%) showed the same band (150 bp) as NILs carrying *Lr34* (Figure 8). The average FDS and AUDPC for these six cultivars were 23.3% and 159, respectively.

DISCUSSION

Wheat leaf rust resistance genes *Lr9*, *Lr19* and *Lr24* are still highly effective against prevalent *Pt* races in China (Yuan *et al.* 2007). However, it is urgent to identify new resistance genes due to the rapid evolution of *Pt* races (Chen *et al.* 1998). Cultivars carrying *Lr19* showed high resistance in the field (Yan *et al*. 2009). But in our selected cultivars from Xinjiang and Anhui provinces, only one cultivar was speculated as carrying *Lr19*. The other two genes *Lr9* and *Lr24* were not detected in any of the tested cultivars. Given that no virulent *Pt* races were reported for these genes currently, a rational usage of these genes in the breeding program will greatly improve wheat leaf rust resistance in these two areas.

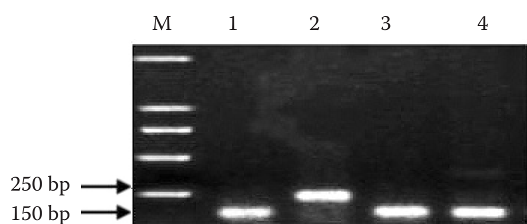

Figure 8. Amplification of specific PCR fragments of the STS marker *Lr34-csLv34*
M – DL2000 marker; 1 – TcLr34; 2 – Yi'nong 16; 3 – Xindong 14; 4 – Xindong 15

Li *et al.* (2010) reported that in the early 1970s, 1BL/1RS translocation with *Lr26* and *Yr9* was introduced into Chinese wheat breeding programs through the introduction of Lovrin 10, Lovrin 13, Predgornaia 2, Kavkaz, and Neuzuch wheat germplasm. Since the gene *Lr26* is linked with *Yr9*, *Pm8* and *Sr31*, it is an excellent resource for wheat resistance to multiple pathogens. However, newly emerged virulent *Pt* races overcoming resistance conferred by *Lr26* were reported in China (Liu & Yang 2004). *Lr26* resistance was overcome by *Pt* in many wheat producing areas of the world including Europe (Bartos *et al*. 1984), the Indian subcontinent (Nayar *et al*. 1991), South Africa (Pretorius *et al*. 1990) and China (Li *et al*. 2010). In this study, there are still 20% of all the collected wheat cultivars carrying this gene.

Lr34 is a slow rusting gene with very good rust resistance at an adult stage in the field (Oelke & Kolmer 2005). *Lr34* had been widely used as priority disease resistance selection in China. Among all the collected wheat cultivars from Xinjiang and Anhui provinces, *Lr34* was detected in 10% of the cultivars. The relatively lower FDS and AUDPC of *Lr34*-carrying cultivars indicate that this gene is still functional to most of the highly virulent *Pt* races in China. The question is whether these 6 cultivars carrying other leaf rust resistance genes should be validated.

In this study, the collected 60 cultivars were inoculated with the mixture of the *Pt* races THTT, THTS, THTQ and PHPS in the field. Interestingly, the numbers of cultivars with high resistance, slow rusting resistance, moderate resistance and susceptibility to virulent leaf rust races approximately obey the normal distribution. We did not observe any highly susceptible cultivars during our screening. The highly resistant and susceptible cultivars are only a small fraction (11.7%) in the tested ones. The majority of the tested cultivars (88.3%) are cultivars with moderate resistance. Further research on the identification of novel resistance genes will greatly improve wheat breeding for leaf rust resistance in China.

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