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## Molecular Mapping of Leaf Rust Resistance Gene *LrL224* in Chinese Wheat Cultivar L224-3

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### Abstract

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Leaf rust, caused by *Puccinia triticina*, is a major wheat disease worldwide. The Chinese wheat cultivar L224-3 showed high resistance to most of *P. triticina* pathotypes in the seedling and adult stage. Identifying and mapping the leaf rust resistance gene(s) in L224-3 is very useful for breeding leaf rust resistant wheat cultivars. In the present study, the wheat cultivar L224-3 and thirty-six lines with known leaf rust resistance genes were inoculated with 15 pathotypes at the seedling stage for gene postulation. A total of 144 F<sub>2,3</sub> lines from the cross L224-3 × Zhengzhou 5389 were inoculated with the pathotype FHBQ for leaf rust genetic analysis at the seedling stage. A total of 1276 SSR (simple sequence repeat) markers and the STS (sequence tagged-site) marker *ω-secali/Glu-B3* were used to test the parents, resistant and susceptible bulks. The polymorphic markers were used to genotype the F<sub>2,3</sub> populations. L224-3 was highly resistant to all *Lr26* avirulent pathotypes, showing the presence of *Lr26* in L224-3. The presence of *Lr26* in L224-3 was also confirmed by the molecular marker *ω-secalin/Glu-B3*. Due to resistance to some *Lr26* virulent pathotypes, L224-3 may carry another resistance gene. Based on the genetic analysis using the pathotype FHBQ with virulence to *Lr26* the resistance of L224-3 was controlled by a single dominant gene, tentatively designated *LrL224*. Four SSR markers (*barc8*, *gwm582*, *wmc419*, and *wmc694*) and one STS marker (*ω-secali/Glu-B3*) on 1B were closely linked to *LrL224*. The two flanking SSR loci were *barc8* and *gwm582*, with the genetic distances of 4.3 and 4.6 cM, respectively. *LrL224* was located on 1BL, and it showed different seedling reactions with other genes on 1B. Therefore *LrL224* is likely to be a new leaf rust resistance gene.

**Keywords:** genetic analysis; molecular marker; *Puccinia triticina*; wheat (*Triticum aestivum* L.)

Leaf rust, caused by *Puccinia triticina* (*Pt*), is a major wheat disease worldwide and can cause significant yield losses wherever wheat is grown in favourable conditions. With global warming, climatic conditions will become more favourable for the occurrence and spread of leaf rust. In China four significant leaf rust epidemics occurred in 1969, 1973, 1975, and 1979 (DONG 2001). In recent years leaf rust has become increasingly important in Northern China and the southern part of the Yellow and Huai Valleys with climate changing (ZHAO *et al.* 2008). In 2012 significant yield losses were recorded in some regions of Gansu,

Sichuan, Shaanxi, Henan, and Anhui (ZHOU *et al.* 2013a). Using leaf rust resistant genes and breeding resistant cultivars are the most efficient, economic and environment-friendly ways of reducing losses caused by leaf rust.

Molecular markers have been widely used in mapping seedling resistance genes for leaf rust. Marker types have changed with advancing technologies and have included restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR), diversity ar-

rays technology (DARt), single nucleotide polymorphisms (SNP), and genotyping-by-sequencing (GBS) (LI *et al.* 2014). SSR markers became the preferred system in the late 1990s owing to codominance, accuracy, high repeatability, high levels of polymorphism, chromosome specificity, and ease of manipulation (RÖDER *et al.* 1998). Molecular markers and bulked segregation analysis (BSA) method could be used for linkage mapping of genetic loci conferring seedling resistance to leaf rust. The number, genetic effects, and locations of genes can be estimated by linkage mapping using molecular markers in segregating populations. To date, more than 100 *Lr* genes have been documented, and 76 have been catalogued (MCINTOSH *et al.* 2016). Some linked molecular markers with variable potential for application in marker-assisted selection are available (LI *et al.* 2014). In addition, our group mapped seven leaf rust seedling resistance genes, *LrZH84*, *LrG98*, and *LrXi* on chromosome 1BL, *LrBi16* and *LrFun* on chromosome 7BL, *LrNJ97* on chromosome 2BL, and *LrZH22* on 2BS (ZHAO *et al.* 2008; CHEN *et al.* 2010; LI *et al.* 2010; ZHANG *et al.* 2011; ZHOU *et al.* 2013; XING *et al.* 2014; WANG *et al.* 2016).

Wheat line L224-3 was developed by Huangfanqu Farm of Henan Province and showed high resistance to most of *Pt* pathotypes in the field. Identifying and mapping the leaf rust resistance gene(s) in L224-3 are very useful for breeding wheat cultivars with durable resistance to leaf rust. In the study molecular markers were tested for mapping a leaf rust resistance gene in L224-3 using a  $F_{2:3}$  population from the cross of L224-3 × Zhengzhou 5389.

## MATERIAL AND METHODS

**Wheat germplasm and *Pt* pathotypes.** Resistant parent L224-3, susceptible parent Zhengzhou 5389 and their  $F_{2:3}$  lines were included in a genetic analysis. Thirty-six lines with known leaf rust resistance genes were kindly provided by the International Maize and Wheat Improvement Center (CIMMYT). Fifteen *Pt* pathotypes used in multi-pathotype comparisons (Table 1) are maintained at the Biological Control Center for Plant Diseases and Plant Pests of Hebei, Hebei Agricultural University, China. These pathotypes are named following the coding system of LONG and KOLMER (1989), with addition of a fourth letter for the reactions to a fourth quartet of differentials ([http://www.ars.usda.gov/SP2/UserFiles/ad\\_hoc/36400500Cerealrusts/pt\\_nomen.pdf](http://www.ars.usda.gov/SP2/UserFiles/ad_hoc/36400500Cerealrusts/pt_nomen.pdf)).

**Seedling test.** L224-3, Zhengzhou 5389, and 36 lines with known *Lr* genes were inoculated with 15 *Pt* pathotypes (Table 1) for comparison of the leaf rust reaction patterns. L224-3, Zhengzhou 5389, 16  $F_1$  plants, and 144  $F_{2:3}$  lines with 30 seedlings each were inoculated with Chinese *Pt* pathotype FHBQ (virulent on Zhengzhou 5389 and avirulent on L224-3 at the seedling stage).

Seedlings were grown in a growth chamber. Inoculations were performed when the first leaves were fully expanded, by brushing urediniospores from fully infected susceptible genotypes onto the seedlings to be tested. Inoculated seedlings were placed in plastic-covered cages at 18°C and 100% relative humidity (RH) for 24 h in darkness. They were then transferred to a growth chamber maintained with 12 h light/12 h darkness at 22–25°C and 70% RH. Infection types (ITs) were scored 10–14 days after inoculation according to the Stakman scale as modified by ROELFS *et al.* (1992). Designations of + and – were used with the 0 to 4 scale to indicate larger and smaller uredinia than normal.

**DNA extraction and bulk preparation.** Genomic DNA was extracted from uninfected  $F_{2:3}$  seedling leaves of all lines (30 plants per  $F_{2:3}$  line as a bulk) including the parents by the CTAB method (SHARP *et al.* 1988). DNA was quantified with a UV spectrophotometer, and diluted to a final concentration of 30 ng/μl prior to further analysis.

Bulked segregant analysis (MICHELMORE *et al.* 1991) was performed to identify molecular markers putatively linked to the leaf rust resistance gene in Zhoumai 22. DNA from 10 homozygous resistant and 10 homozygous susceptible  $F_{2:3}$  lines based on the seedling test were mixed in equal amounts to form resistant and susceptible bulks. The DNA bulks and samples from the two parents were tested for polymorphism using SSR primers.

**Marker analysis.** A total of 1276 SSR markers available in GrainGenes 2.0 (<http://wheat.pw.usda.gov>) and a STS (sequence tagged-site) marker *ω-secali/Glu-B3* were used to search for polymorphisms between the parents of the mapping populations. The SSRs used in this study were BARC (SONG *et al.* 2002), CFA and CFD (SOURDILLE *et al.* 2004), WMC (GUPTA *et al.* 2002) and GWM (RÖDER *et al.* 1998) series.

Markers that showed similar patterns of polymorphism between the bulks and parents were used to assay the mapping populations. SSR analysis followed BRYAN *et al.* (1997) with minor modifications.

PCR were performed in volumes of 20 μl containing 1.0 U Taq DNA polymerase (Zexing Biotechnology

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Table 1. Seedling infection types of 36 control wheat lines with known leaf rust resistance and the two cultivars to 15 *Puccinia triticina* pathotypes

line	gene	Pathotypes															
		PH KS	MH JS	FH DQ	FG BQ	FH BR	FH BQ	FG BR	PH JL	FH DR	FG DQ	FH DS	TH JP	TG TT	PH GP	TH JC	
RL6003	<i>Lr1</i>	4	4	;;	;;	;;	;;	;;	4	0	;;	0	4	4	4	4	
RL6016	<i>Lr2a</i>	;;	;;	1+	;;	;;	1	1	3	;;	;;	2	3	3	;;	4	
RL6047	<i>Lr2c</i>	4	1	4	4	4	4	4	4	4	4	4	4	4	4	4	
RL6002	<i>Lr3</i>	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
RL6010	<i>Lr9</i>	;	;	;	;	0;	0	0	;	0	0	;	;	;	0	;	
RL6005	<i>Lr16</i>	4	4	4	4	4	4	3+	3+	4	4	4	4	4	3	4	
RL6064	<i>Lr24</i>	;;1	;	;;	;;	;;	;;	;;	;	;	;	;	;	;	;	;	
RL6078	<i>Lr26</i>	4	4	4	1	4	4	;	4	4	1	4	4	2	4	4	
RL6007	<i>Lr3ka</i>	X	X	;	;	;	;	1	1	;	;	;	1	4	;	X	
RL6053	<i>Lr11</i>	4	4	1	;	;	1+	2	3+	1	1	2	4	3+	4	4	
RL6008	<i>Lr17</i>	4	3+	3+	2	2	2	2+	4	3+	4	4	4	4	2+	4	
RL6049	<i>Lr30</i>	3C	1	1	;	;	;	;	1	;	;	;	;	4	;	1	
RL6051	<i>LrB</i>	3+	4	4	4	3+	4	4	3+	4	4	4	4	4	4	X	
RL6004	<i>Lr10</i>	3	3	4	4	4	4	4	2	4	4	4	2+	4	1	X	
RL6013	<i>Lr14a</i>	4	4	X	X	X	X	X	X	X	2	4	4	4	3+	X	
RL6009	<i>Lr18</i>	1	1+	2	2	4	2	4	1+	4	2+	2	4	3+	3C	3	
RL6019	<i>Lr2b</i>	1	0;	4	;	3	3+	2	4	3	3+	3+	2	4	3C	4	
RL6042	<i>Lr3bg</i>	4	4	4	4	4	3+	4	4	4	4	4	4	4	4	4	
RL4031	<i>Lr13</i>	3	4	4	3	3	4	4	3	3	2	3+	4	4	4	4	
RL6006	<i>Lr14b</i>	4	4	4	4	4	4	4	4	4	4	4	X	4	X	4	
RL6052	<i>Lr15</i>	1	;	;	;	;	;	;	4	1	;	;	4	3+	4	4	
RL6040	<i>Lr19</i>	0	0	;	0	0	;	0	0	0	0	;	0	0	0	;	
RL6092	<i>Lr20</i>	4	4	;	;	;	;	0	;	;	;	;	4	1	4	;	
RL6043	<i>Lr21</i>	4	2	2	;	2+	3	2	;	1	;	1+	;	3	1	1	
RL6012	<i>Lr23</i>	4	4	4	3+	3+	4	3+	1	4	4	4	4	4	3+	4	
RL6079	<i>Lr28</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
RL6080	<i>Lr29</i>	0	0	0	0	;	0	0	;	;	0	3+	4	;	0	0	
RL6057	<i>Lr33</i>	3	4	3+	3+	3+	4	2+	3C	3+	3	4	4	4	3+	3+	
E84018	<i>Lr36</i>	4	2	1+	;	2	2	1	1	2	2+	3	2+	3+	2+	3+	
KS86NGRC02	<i>Lr39</i>	;	;1	;	;	;	;	;	;	;	;	;	;	;	;	;	
KS91WGRC11	<i>Lr42</i>	;	;	;	0	0	;	;	;	1	;	;	0	;	0	1	
RL6147	<i>Lr44</i>	1	;	4	4	4	4	4	1	4	4	4	;	1+	;1	1	
RL614	<i>Lr45</i>	4	4	4	4	4	4	4	;	4	4	4	4	;	;	;	
PAVON76	<i>Lr47</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
C78.5	<i>Lr51</i>	;	;	;	;	1	;	0	;	;	;	;	0	;	;	;	
-98M71	<i>Lr53</i>	;	0	0	0	0	0	0	;	0	0	0	0	0	0	0	
L224-3	<i>LrL224</i>	3	3+	;1	0	1	1	0	3	2	;	;	3	2	2+	;	
Zhou 8425B	<i>LrZH84</i>	4	3+	2	0	3c	4	0	4	3+	0	2	4	2	4	4	
Zhengzhou 5389	+	4	4	4	4	3+	4	4	4	4	4	4	4	4	4	4	

Infection types follow 0–4 scale (ROELFS *et al.* 1992): 0 = no uredinia or other macroscopic signs of infection; ; = no uredinia, but hypersensitive necrotic or chlorotic flecks of varying size; 1 = small uredinia surrounded by necrosis; 2 = small to medium uredinia surrounded by green islands; X = random distribution of variable-sized uredinia on single leaf with a pure culture; 3, 4 = medium to large uredinia without chlorosis or necrosis; + = uredinia somewhat larger than normal for the infection type; – = uredinia somewhat smaller than normal for infection type; C = more chlorosis than normal for infection type

Table 2.  $F_{2:3}$  phenotype, genotype and the corresponding alleles at SSR loci *Xbarc8* and *Xgwm582* in the cross of L224-3 × Zhengzhou 5389

Marker loci	$F_{2:3}$ phenotype	$F_{2:3}$ genotype	Allele		
			A	H	B
<i>Xbarc8</i>	resistant (98)	<i>RR</i> (35)	35	0	0
		<i>Rr</i> (63)	4	56	3
	susceptible (46)	<i>rr</i> (46)	0	4	42
<i>Xgwm582</i>	resistant (98)	<i>RR</i> (35)	35	0	0
		<i>Rr</i> (63)	4	58	1
	susceptible (46)	<i>rr</i> (46)	0	8	38

*RR* – homozygous resistant; *Rr* – segregating; *rr* – homozygous susceptible; *A* – homozygous for the L224-3 allele, *B* – homozygous for the Zhengzhou 5389 allele, *H* – heterozygous

Co. Ltd, Beijing, China), 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, pH 8.3), 200 μM of each dNTP, 6 pmol of each primer and 60 ng of template DNA. The PCR conditions were as follows: denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 50–61°C (depending on the primer pair) for 1 min, 72°C for 1 min and a final extension for 10 min at 72°C. PCR products were mixed with 8 μl of formamide loading buffer (98% formamide, 10 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cynol, pH 8.0). Each sample (5 μl) was loaded on 6% non-denaturing polyacrylamide gels, and run at 300 V for approximately 1 h and visualized by silver staining (BASSAM *et al.* 1991).

**Linkage analysis and map construction.** Goodness-of-fit of observed and expected segregation ratios was evaluated by chi-squared ( $\chi^2$ ) tests. Linkage analysis was performed using the software MapManager QTXb20 (MANLY *et al.* 2001) and recombination values were converted to centiMorgans using the Kosambi mapping function (KOSAMBI 1944). The chromosomal assignments of the linkage groups

were based on published wheat maps (SOMERS *et al.* 2004) and GrainGenes (<http://wheat.pw.usda.gov>).

## RESULTS

**Seedling reactions of L224-3.** In seedling tests with 15 *P. triticina* pathotypes (Table 1), L224-3 was resistant to four pathotypes avirulent to *Lr26*, indicating that *Lr26* was present in L224-3. The presence of *Lr26* in L224-3 was also confirmed by molecular marker  $\omega$ -*secalin/Glu-B3* (DE FROIDMONT 1998; CHAI *et al.* 2006). L224-3 was postulated to also possess other unknown genes according to their low reactions with certain *Lr26*-virulent pathotypes (Table 1).

**Inheritance of seedling leaf rust resistance in L224-3.** In the seedling test with pathotype FHBQ with virulence to *Lr26*, L224-3 was resistant with infection type (IT) 1, whereas Zhengzhou 5389 was susceptible with IT 4.  $F_1$  plants were resistant with IT 1 to 2. Of 144  $F_{2:3}$  lines tested with FHBQ, 35 lines were homozygous resistant, 63 segregated and 46 were homozygous susceptible, fitting a 1:2:1 ratio ( $\chi^2_{1:2:1} = 3.93, 2df, P > 0.10$ , Table 2), indicating a single dominant gene for resistance besides *Lr26*. The dominant gene in L224-3 was tentatively designated as *LrL224*, conferred resistance to the pathotype FHBQ.

**Linkage analysis and genetic map.** Of 1276 SSR markers and 1 STS marker  $\omega$ -*secalin/Glu-B3*, five (*barc8*, *gwm582*, *wmc419*, *wmc694* and  $\omega$ -*secalin/Glu-B3*) on chromosome 1B showed polymorphisms between the resistant and susceptible bulks, indicating that *LrL224* was located on chromosome 1B. The five polymorphic markers were then screened on DNA from the 144  $F_{2:3}$  lines previously tested with leaf rust. The results showed that resistance gene *LrL224* was closely linked to the five loci with genetic distances ranging from 4.3 cM to 14.3 cM (Figure 3). The two closest flanking SSR loci were *Xbarc8-1RS* and *Xgwm582-1BL* with genetic distances of 4.3 cM and 4.6 cM (Figures 1–3; Table 2), respectively.

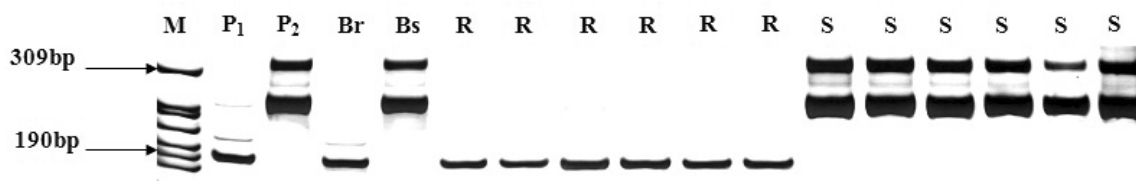


Figure 1. The result of Native-PAGE of L224-3, Zhengzhou 5389, resistant and susceptible bulks, and their  $F_{2:3}$  lines by the marker *Xbarc8*; M – molecular marker PBR322; P1 – resistant parent L224-3; P2 – susceptible parent Zhengzhou 5389; Br – resistant bulk; Bs – susceptible bulk; R – resistant  $F_{2:3}$  lines plants; S – susceptible  $F_{2:3}$  lines plants



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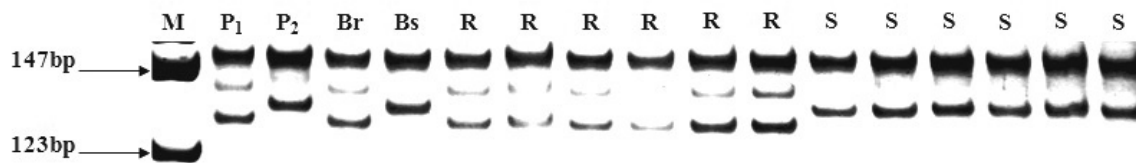


Figure 2. The result of Native-PAGE of L224-3, Zhengzhou 5389, resistant and susceptible bulks, and their  $F_{2.3}$  lines by the marker *Xgwm582*; M – molecular marker PBR322; P1 – resistant parent L224-3; P2 – susceptible parent Zhengzhou 5389; Br – resistant bulk; Bs – susceptible bulk; R – resistant  $F_{2.3}$  lines plants; S – susceptible  $F_{2.3}$  lines plants

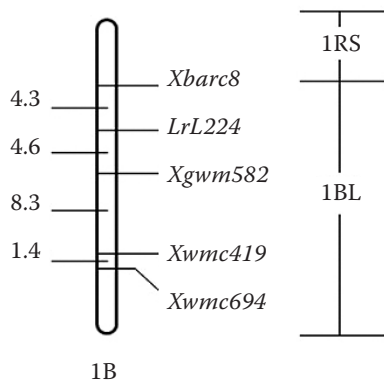


Figure 3. Linkage map of four molecular markers and wheat leaf rust resistance gene *LrL224* on chromosome 1B

## DISCUSSION

**Comparison of *LrL224* with leaf rust resistance genes located on chromosome 1B.** Nine leaf rust resistance genes *Lr26*, *Lr33*, *Lr44*, *Lr46*, *Lr51*, *Lr55*, *LrZH84*, *LrXi* and *LrG98* are located on chromosome 1B. *Lr26* was originally derived from *Secale cereale*, *Lr33* was derived from a common wheat line, *Lr44* was derived from spelt wheat, and *Lr51* was present in alien chromosome segments derived from *Aegilops speltoides* (HIEBERT *et al.* 2005). In the present seedling tests, *Lr33* and *Lr44* performed susceptible to pathotypes FHDQ, FHBR, FHBQ and FHDS, indicating that *LrL224* is different from *Lr33* and *Lr44*. Based on the seedling tests with 15 *P. triticina* pathotypes in this study, *Lr51* was highly resistant to all pathotypes, whereas *LrL224* was susceptible to the part of the pathotypes, indicating that *LrL224* is different from *Lr51*. *Lr46* is an adult-plant resistance gene located in the terminal region of the long arm of wheat chromosome 1B (SINGH *et al.* 1998; ROSEWARNE *et al.* 2006). *Lr55* is present in alien chromosome segments derived from *Elymus trachycaulis* (MCINTOSH *et al.* 2005), so it is different from *LrL224*. Three resistance genes *LrZH84*, *LrXi* and *LrG98* on 1B were reported previously in our group (ZHAO *et al.* 2008; LI *et al.* 2010; CHEN *et al.* 2010), and all of them are near to *LrL224*.

ZHOU *et al.* (2013b) reported that *LrXi*, *LrG98*, and *LrZH84* were likely to be allelic or closely linked. In the present seedling tests, Zhou8425B was susceptible to pathotypes FHBQ avirulent to *LrL224*, suggesting *LrL224* is different from *LrZH84*. The genetic relationship between *LrL224* and these genes should be tested in the future.

**Adult plant resistance of L224-3.** To identify adult plant resistance (APR) present in L224-3 the  $F_{2.3}$  population from L224-3 × Zhengzhou 5389 was also grown in the field for leaf rust evaluation. Leaf rust epidemics were initiated by inoculating *Pt* pathotypes virulent to L224-3 at the seedling stage. The result showed that *LrL224* was susceptible with IT 4 in the field, but it had a low maximum disease severity (MDS) score of 0–5%, indicating L224-3 showed a high level of APR to Chinese *Puccinia triticina* pathotypes in the field. Now QTL mapping for APR gene in the population is underway. Seedling gene and APR gene present in L224-3 as well as their closely linked molecular markers will be potentially useful for improving leaf rust resistance in wheat breeding programs in China.

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