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An AFLP marker linked to the leaf rust resistance gene *LrBi16* and test of allelism with *Lr14a* on chromosome arm 7BL

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ABSTRACT

Leaf rust (LR), caused by *Puccinia triticina*, is one of the most widespread diseases of common wheat (*Triticum aestivum* L.) worldwide. The LR resistance gene *LrBi16* has been mapped on chromosome arm 7BL in Chinese wheat cultivar Bimai 16 and was closely linked to SSR loci *Xcfa2257* and *Xgwm344* with genetic distances of 2.8 cM and 2.9 cM, respectively. In the present study, a total of 304 AFLP primer pairs were used to screen Bimai 16 and Thatcher and resistant and susceptible DNA bulks. The polymorphic AFLP marker *P-ATT/M-CGC*$_{173\ bp}$ was used to genotype F_2 and F_3 populations to identify markers more closely linked to *LrBi16*. Marker *P-ATT/M-CGC*$_{173\ bp}$ was tightly linked to *LrBi16* with a genetic distance of 0.5 cM. As *LrBi16* was mapped near the *Lr14a* locus, 809 F_2 plants from the Bimai 16/RL6013 (*Lr14a*) cross were inoculated with the *Pt* pathotype FHNQ to test the allelism of *Lr14a* and *LrBi16*. All of the F_2 plants were resistant to FHNQ (IT between; and 2), suggesting that *Lr14a* and *LrBi16* are allelic.

1. Introduction

Leaf rust (LR), caused by *Puccinia triticina* (*Pt*), is one of the most important and widespread diseases in wheat (*Triticum aestivum* L.). It occurs in a wide range of climates wherever wheat is grown and causes yield losses up to 65% under favorable conditions [1]. In China LR has historically been important only in the southwest and northeast regions [2], but with increased planting densities and changing management practices it has become increasingly important in most major wheat-producing areas. Destructive epidemics of LR occurred in 1969, 1973, 1975, and 1979 in China [2], and yield losses occurred in regions of Gansu, Sichuan, Shaanxi, Henan and Anhui provinces in 2012 [3]. Resistant cultivars are the most efficient, economical, and environmentally safe way to manage LR.

Resistance to LR can be classified into two types, qualitative resistance conferred by single resistance genes (also termed as major, seedling, or race specific resistance) and quantitative resistance, mediated by multiple genes or quantitative trait loci

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(QTL) (also termed as adult plant resistance, race nonspecific, or slow rusting resistance). To date, more than 100 LR resistance genes have been described in wheat, with 72 formally designated genes [4]. Most of these genes are qualitative and interact with the pathogen in a gene-for-gene fashion [5]. This type of gene is rapidly overcome by the pathogen. Only *Lr9*, *Lr19*, *Lr24*, and *Lr38* are currently effective against the prevalent Chinese *Pt* pathotypes [6,7]. Thus, it is important to identify and use new resistance resources to control the dynamic and rapidly evolving pathogen population [8].

Molecular markers, including RFLP, RAPD, SSR, AFLP, and EST, have been widely used to map LR resistance genes in different genetic populations. To date, 46 LR genes have been mapped on various chromosomes using molecular markers [9]. However, most markers are too far from resistance genes for reliable use in breeding programs. It is thus important to identify more closely linked or gene-based molecular markers for marker-assisted selection (MAS). The AFLP technique has been widely used in the construction of high-density genetic maps, enabling several LR genes to be finely mapped in wheat using this type of marker combined with bulked segregant analysis (BSA) [10–15].

Bimai 16, released in 2004 by the Bijie Agricultural Science Research Institute, Guizhou province, has high levels of resistance to leaf rust, stripe rust (caused by *Puccinia striiformis* f. sp. *tritici*) and powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*) under field conditions [16,17]. A dominant LR resistance gene, *LrBi16*, on chromosome arm 7BL is flanked by SSR markers *Xcfa2257* and *Xgwm344* at genetic distances of 2.8 cM and 2.9 cM, respectively [18]. *LrBi16* is very close to the *Lr14* locus, with named resistance alleles *Lr14a* and *Lr14b* that were combined in a rare recombination event [19]. The objectives of the present study were to identify molecular markers more closely linked to *LrBi16* using AFLP markers and to determine the allelic relationship between *LrBi16* and *Lr14a*.

2. Materials and methods

2.1. Plant materials and Pt pathotypes

A total of 359 F_2 plants and 298 F_3 lines were derived from a cross of the resistant parent Bimai 16 (pedigree: 8513-1624/Ji 1002) with the susceptible parent Thatcher. The Chinese *Pt* pathotype, FHTT, was used to inoculate the genetic materials. Phenotypic and genotypic data for the F_2 and F_3 populations were derived from our previous study [18]. A total of 809 F_2 plants from Bimai 16/RL6013 (*Lr14a*) and the Chinese *P. triticina* pathotype FHNQ were employed to test the allelism of *LrBi16* with *Lr14a*. The Thatcher near-isogenic line, RL6013 with *Lr14a*, was kindly provided by the USDA—ARS Cereal Disease Laboratory, University of Minnesota, St. Paul, USA. The two pathotypes were designated based on the coding system of Long and Kolmer [20] with addition of a fourth letter for the reactions of a fourth set of differentials (http://www.ars.usda.gov/SP2UserFiles/ad_hoc/36400500Cerealrusts/pt_nomen.pdf). All the wheat germplasm and *Pt* pathotypes are maintained at the Biological Control Center for Plant Diseases and Plant Pests of Hebei, Hebei Agricultural University, Baoding, China.

Table 1 – AFLP adapters developed in this study.

Adapter	Sequence (5′–3′)
Pst I	F: CTCGTAGACTGCGTACATGCA; R: TGTACGCAGTCTAC
Mse I	F: GACGATGAGTCCTGAG; R: TATCAGGACTCAT

2.2. Allelism analysis between LrBi16 and Lr14a

Pathotype FHNQ (avirulent to both *LrBi16* and *Lr14a*) was used to inoculate the F_2 population from the Bimai 16/RL6013 (*Lr14a*) cross for the allelism test between *Lr14a* and *LrBi16*. The F_2 plants were grown in a growth chamber. Inoculation was performed when the first leaves were fully expanded, by brushing urediniospores from fully infected susceptible plants of Zhengzhou 5389 onto the F_2 plants. Inoculated plants were placed in plastic-covered cages, incubated at 15 °C and 100% relative humidity (RH) for 24 h in darkness, and then transferred to a growth chamber programmed for 12 h light/12 h darkness at 18 to 22 °C and 70% RH. Infection types (ITs) were scored 10 to 14 days after inoculation according to the Stakman scale, modified by Roelfs et al. [21].

2.3. AFLP analysis

A total of 304 AFLP primers were used to screen the parents and resistant (Br) and susceptible DNA bulks (Bs). Markers with consistent polymorphism between the parents and the bulks were used to analyze the entire F_2 and F_3 populations. AFLP analysis was performed following Zhang et al. [14]. Genomic DNA was digested with *Pst* I and *Mse* I (Table 1), ligated with adapters, and then pre-amplified with primers containing one selective nucleotide (Table 2). The samples were diluted 20-fold with ddH_2O and stored at 4 °C. Selective amplification was achieved by primers with three selective nucleotides (Table 3). Amplification was performed in a T-gradient thermal cycler PCR (Bio-Metra, Göttingen, Germany). PCR-amplified AFLP products (5 μL) were mixed with 1 μL of loading buffer (98% formamide, 10 mmol L^{-1} EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, pH 8.0), and loaded on 6% denaturing polyacrylamide gels. The gels were run at 80 W for approximately 3 h and visualized by silver staining [22].

2.4. Linkage analysis

Linkage analysis using the combined previous SSR and current AFLP results was performed using the software MapManager QTXb20 [23] and recombination values converted to centiMorgans using the Kosambi mapping function [24].

Table 2 – Pre-amplification primers.

Primer	Sequence (5′–3′)
Pst I + A	GACTGCGTACATGCAGA
Pst I + T	GACTGCGTACATGCAGT
Mse I + C	GATGAGTCCTGAGTAAC
Mse I + G	GATGAGTCCTGAGTAAG

Table 3 – Primers used for selective amplification.

Primers of *Pst* I + 3 (5′–3′)	Primers of *Mse* I + 3 (5′–3′)
Pst I-AAC: GACTGCGTACATGCAGAAC	*Mse* I-CAA: GATGAGTCCTGAGTAACAA
Pst I-AAG: GACTGCGTACATGCAGAAG	*Mse* I-CAC: GATGAGTCCTGAGTAACAC
Pst I-AAT: GACTGCGTACATGCAGAAT	*Mse* I-CAG: GATGAGTCCTGAGTAACAG
Pst I-ACA: GACTGCGTACATGCAGACA	*Mse* I-CAT: GATGAGTCCTGAGTAACAT
Pst I-ACG: GACTGCGTACATGCAGACG	*Mse* I-CTA: GATGAGTCCTGAGTAACTA
Pst I-ACC: GACTGCGTACATGCAGACC	*Mse* I-CTC: GATGAGTCCTGAGTAACTC
Pst I-ACT: GACTGCGTACATGCAGACT	*Mse* I-CTG: GATGAGTCCTGAGTAACTG
Pst I-AGA: GACTGCGTACATGCAGAGA	*Mse* I-CTT: GATGAGTCCTGAGTAACTT
Pst I-AGC: GACTGCGTACATGCAGAGC	*Mse* I-CGA: GATGAGTCCTGAGTAACGA
Pst I-AGG: GACTGCGTACATGCAGAGG	*Mse* I-CGC: GATGAGTCCTGAGTAACGC
Pst I-AGT: GACTGCGTACATGCAGAGT	*Mse* I-CGG: GATGAGTCCTGAGTAACGG
Pst I-ATA: GACTGCGTACATGCAGATA	*Mse* I-CGT: GATGAGTCCTGAGTAACGT
Pst I-ATC: GACTGCGTACATGCAGATC	*Mse* I-GGA: GATGAGTCCTGAGTAAGGA
Pst I-ATG: GACTGCGTACATGCAGATG	*Mse* I-GGT: GATGAGTCCTGAGTAAGGT
Pst I-ATT: GACTGCGTACATGCAGATT	*Mse* I-GAC: GATGAGTCCTGAGTAAGAC
Pst I-TAG: GACTGCGTACATGCAGTAG	*Mse* I-GAG: GATGAGTCCTGAGTAAGAG
Pst I-TGA: GACTGCGTACATGCAGTGA	
Pst I-TCT: GACTGCGTACATGCAGTCT	
Pst I-TCA: GACTGCGTACATGCAGTCA	

2.5. Cloning of PCR fragments amplified from the AFLP markers

The isolation of PCR fragments followed Xu et al. [25]. Briefly, the PCR products from the AFLP marker were excised from dried gels. Spliced gels containing the amplification products were transferred to a PCR tube and eluted twice with 200 μL of 1× TE buffer (pH 8.0) for 30 min and once with 200 μL of ddH_2O for 30 min. The gel was then soaked in 50 μL of ddH_2O and kept in a PCR thermocycler at 95 °C for 10 min to release the DNA from the gel. After the gel debris was spun down by centrifuging at 3000 r min^{-1} for 10 min, the supernatant was used as template DNA for PCR amplification, using the same AFLP primers and PCR conditions.

3. Results

3.1. Seedling allelism test

Both Bimai 16 and RL6013 were resistant to pathotype FHNQ with IT 2. A total of 809 F_2 plants derived from Bimai 16/RL6013 (*Lr14a*) were inoculated with pathotype FHNQ. Both parents and all the F_2 plants were resistant to FHNQ with ITs ranging from fleck (;) to 2, indicating that *Lr14a* and *LrBi16* were allelic or closely linked genes.

3.2. AFLP marker of LrBi16

Among 304 AFLP markers, only *P-ATT/M-CGC*$_{173\ bp}$ was polymorphic between the parents as well as Br and Bs. This marker was then used to genotype the entire F_2 population. Marker *P-ATT/M-CGC*$_{173\ bp}$ was closely linked to *LrBi16* at a genetic distance of 0.5 cM (Figs. 1, 2) and was more closely linked to *LrBi16* than was marker *Xgwm344* reported in our previous study [18]. The marker was also used to genotype the 298 F_3 lines, and was found to lie 0.7 cM from *LrBi16*.

3.3. Cloning and sequencing of the AFLP specific DNA fragment

The size of the re-amplified band was the same as that of the AFLP-specific DNA fragment after recovery and a specific band of 173 bp was sequenced (Fig. 3). Although STS primers were designed, no polymorphism was found between the parents and the bulks (Fig. 3). This AFLP could not be converted to an STS/SCAR marker, owing to the small fragment size.

4. Discussion

4.1. Comparison between LrBi16 and other wheat leaf rust resistance genes on chromosome arm 7BL

Leaf rust resistance genes located on chromosome arm 7BL include *LrBi16*, *Lr14a*, *Lr14b* [19], *Lr68* [26], and *LrFun* [9]. *Lr68* is an adult plant resistance gene closely linked to SSR marker *Xgwm146* [26], which is also linked to *LrBi16* [18], whereas *LrBi16* confers seedling resistance with ITs from 1 to 2 [18]. *Lr14a*, originating in *T. turgidum*, was mapped on 7BL [27], and like *LrBi16*, is linked to SSR marker *Xgwm344-7B* [18]. However,

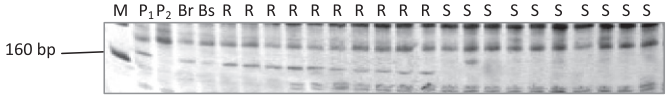

Fig. 1 – Electrophoresis of PCR products amplified with AFLP marker *P-ATT/M-CGC*$_{173\ bp}$ on a polyacrylamide gel. M: *PBR322/MspI* marker; P_1: resistant parent Bimai 16; P_2: susceptible parent Thatcher; Br: resistant bulk; Bs: susceptible bulk; R: resistant F_2 plants; and S: susceptible F_2 plants.

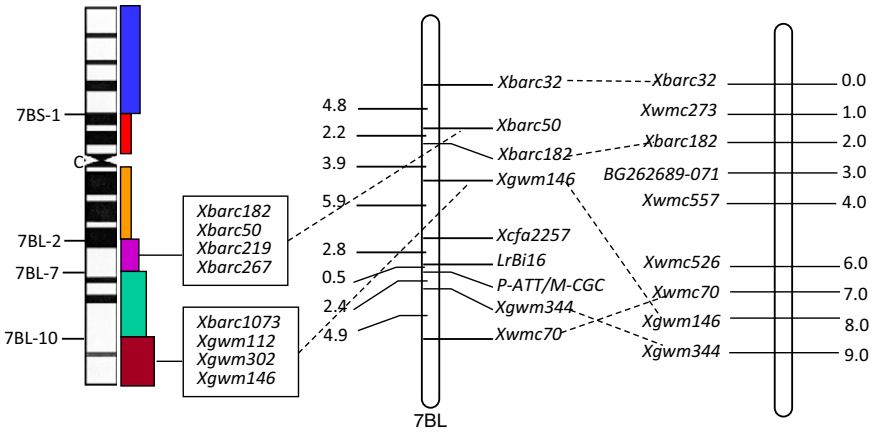

Fig. 2 – Deletion bin map of chromosome 7B [30] (left) and linkage maps of leaf rust resistance gene region and 8 loci based on 359 F_2 plants of Bimai 16/Thatcher (right). Locus names and corresponding locations are indicated on the right and map distances in centiMorgans are shown on the left (center panel) and compared with previously [31] published map (right panel).

*GACTGCGTACATGCAGATTT*GAATGAATAGGATTGGATCTAGGACACAACTAGCTCGC
CTTCTCGGTCATTCAACGGTAACCACTGCTCCAGGGACATATTCTTCTTCCAGATCCCCG
GGTCGTAAAGCTTTGAAACAAAGGGAAAAAGAATGAGCGTTACTCAGGACTCATC

Fig. 3 – Specific band sequence of *P-ATT/M-CGC*$_{173\ bp}$. Primer sequences are shown in shadow part.

RL6013 (Thatcher + *Lr14a*) was susceptible to most pathotypes, including two that are avirulent to *LrBi16* [18], indicating that *LrBi16* is different from *Lr14a*. A test of allelism involving 809 F_2 plants revealed no recombinants between *Lr14a* and *LrBi16*, indicating that the genes are allelic or tightly linked. In an earlier study *Lr14b* was very closely linked rather than allelic to *Lr14a* [19]. Another seedling resistance gene, *LrFun*, was also mapped near *LrBi16* on chromosome arm 7BL, but *LrBi16* differed from *LrFun* in reactions to a panel of *Pt* cultures [9]. However, the relationship between *LrBi16* and *LrFun* awaits confirmation by further allelic studies. Several LR resistance genes have been mapped in this region, suggesting that it is an important region for leaf rust resistance.

4.2. Development of STS/SCAR markers from the AFLP marker

Although AFLP technology can detect many genetic loci, it has limitations when applied to MAS, owing to the production of many nonspecific bands and corresponding difficulty in scoring. STS markers developed from AFLP products are more specific and more easily used in MAS. However, not all AFLP can be successfully converted to STS markers, owing to the small size of DNA fragments amplified by AFLP and a lack of polymorphisms in restriction sites between genotypes [28]. In addition, different AFLP fragments with the same size may co-migrate on a gel, and a target polymorphic band may contain contaminating fragments from different bands [11,29], again complicating the development of an STS marker from an AFLP product. In the present study, the specific fragment linked to *LrBi16* was cloned and sequenced, and primers were designed according to the sequence, but no polymorphism was found between resistant and susceptible bulks. This result was attributed to either the small size of the AFLP fragment or a lack of polymorphism at the restriction site.

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